

**MOLECULAR EPIDEMIOLOGY OF BOVINE
TUBERCULOSIS IN TANZANIA**

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ABSTRACT

A study on molecular epidemiology of bovine tuberculosis in man and cattle in Tanzania was carried with two components. The first component was based on field investigation of tuberculosis in cattle and man in Arusha region, in the north and in the Usangu Plains in the Southern Highlands of Tanzania. The second component involved laboratory analysis of mycobacterial strains acquired from field study by both conventional and molecular biology techniques.

In field work, a total of 6383 cattle were tuberculin tested, and 911 (14.3%) were classified as reactors. 841 of 6383 cattle examined were destined for slaughter and 225 (26.8%) were found to have visible lesions. A total of 1719 pooled lymph node samples collected from slaughter cattle in the study area (Arusha n=1068 and the Usangu Plains n=651) were cultured and 4.0% yielded mycobacteria. 80 of the samples from the Usangu Plains came from cattle classified as reactors (Table 3.9), while samples from Arusha had no accompanying tuberculin test results. Among the isolates 72.6% were classified as *M. bovis* and the rest as atypical mycobacteria. Of the 805 milk samples cultured (14% were from reactor cattle), only two (0.3%) yielded *M. bovis*, the remaining 36 isolates were atypical mycobacteria (Table 3.10). Its only two isolates of atypical mycobacteria which came from reactor cattle.

Regarding human specimens, a total of 53 lymph node biopsies were submitted for culture and 21 (39.6%) were positive for mycobacteria. Six isolates were identified as *M. bovis*, and the remaining 15 were *M. tuberculosis*. Of 96 sputum samples which were collected, mycobacteria species were recovered from 23 but only one (4.3%) was classified as *M. bovis*. Sixteen (69.1%) isolates were identified as *M. tuberculosis*, and the rest were atypical mycobacteria. Epidemiological data revealed that *M. bovis* was more prevalent in people with cattle contact than in other occupations (Table 3.13)

The IS986 and *mtp40* multiplex PCR developed in the course of this study was able to differentiate *M. bovis* from *M. tuberculosis*. DNA fingerprinting of all the strains cultured was carried out using restriction fragment length polymorphism (RFLP) and spoligotyping techniques. The copy number of IS986 amongst strains of *M. bovis* from cattle ranged from one to six, while those from man had five to 15. There was

similarity of DNA fingerprints among some of the strains from cattle and man as determined by the three typing techniques (Figures 6.2, 6.5 and 6.9). *M. tuberculosis* strains were found to belong to three clusters by IS986 RFLP, with one cluster containing over 60% of the strains.

Intersegment PCR, a molecular typing technique developed in the current study was able to differentiate strains but the results were influenced by the concentration of template DNA. A DNA fragment apparently found only in *M. bovis* and absent in *M. tuberculosis* and other mycobacteria was identified by the RAPD PCR techniques. This fragment was cloned, sequenced and its DNA sequence was found to match a *M. tuberculosis* cosmid, which also matched *rfbE* gene of *Yersinia enterocolitica*. Specificity testing revealed hybridization to *M. tuberculosis* as well.

The findings of the above studies have showed the existence of *M. bovis* infection in man and cattle in Tanzania. The study has also shown the zoonotic importance of infection in the two populations which necessitates a veterinary/medical approach to the control of the disease in Tanzania. Furthermore, it has been shown that molecular biology techniques are essential epidemiological tools in studies of zoonotic conditions such as tuberculosis. The study was unable to find a specific DNA element for *M. bovis*. This observation concurs with others which have found 100% homogeneity between species of the *M. tuberculosis* complex.

DECLARATION

This thesis was composed by myself. I declare that all the work presented in this thesis was performed by myself and whenever work was performed by other people in the context of the larger project, I was fully involved in its design and analysis. The work described formed the major part of the Bovine Tuberculosis in the Tropics (BTB) project of which I was a principal investigator, but the contributions made by other participants of the project are fully acknowledged in the text. This work has not been and is not currently submitted for the award of any other qualification.

Rudovick Reuben Kazwala

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CONTENTS

Abstract	ii
Declaration	iv
Acknowledgement	v
Contents	vii
List of figures	xii
List of tables	xvi
Abbreviations	xix

CHAPTER 1: INTRODUCTION

1.1. REVIEW OF THE LITERATURE	2
1.1.1. History of tuberculosis	2
1.1.2. Nomenclature	5
1.1.3. Bacteriology	7
1.1.4. Molecular detection, identification and typing	11
1.1.5. Bovine tuberculosis in cattle	19
1.1.6. Diagnosis	21
1.1.7. Epidemiology of bovine tuberculosis	28
1.1.8. Zoonotic implications of <i>M. bovis</i>	35
1.1.9. Control of <i>M. bovis</i> infection in cattle and man	42
1.1.9.1. Control of bovine tuberculosis in cattle	42
1.1.9.2. Control of <i>M. bovis</i> infection in man	45
1.2. BOVINE TUBERCULOSIS IN TANZANIA	46
1.2.1. Background information	46
1.2.2. Epidemiology of bovine tb in Tanzania	47
1.2.3. Zoonotic implication of bovine tb in Tanzania	49
1.3. Objectives of the study	53

CHAPTER 2: GENERAL MATERIALS AND METHODS

2.1. FIELD WORK	55
2.1.1. Study areas	55
2.1.2. Study populations	56
2.1.3. Collection of specimens	59
2.1.4. Tuberculin testing of cattle	61
2.1.5. Epidemiological data collection and analysis	62
2.2. LABORATORY WORK	63
2.2.1. Bacteriology	63
2.2.2. Molecular biology techniques	67
2.2.2.1. DNA extraction	67
2.2.2.2. Determination of quality and quantity of DNA	69
2.2.2.3. Molecular identification - Polymerase chain reaction (PCR)	69
2.2.2.4. Molecular typing - DNA Fingerprinting	76
2.2.2.5. Spoligotyping	82
2.2.2.6. Cloning of RAPD PCR fragments	85
2.2.2.7. Sequencing of cloned <i>f2b</i> and <i>f3b</i> DNA fragments	86
2.2.2.8. Sequencing gel electrophoresis	87

CHAPTER 3: EPIDEMIOLOGY OF BOVINE TUBERCULOSIS IN MAN AND CATTLE IN TANZANIA

3.1. INTRODUCTION	89
3.1.1. Bovine tuberculosis in cattle	89
3.1.2. Zoonotic importance of <i>M. bovis</i>	89
3.1.3. Aims of the study	90
3.2. RESULTS	91
3.2.1. Bovine tuberculosis in cattle	91
3.2.1.1. Tuberculin testing	91
3.2.1.2. Post-mortem examinations	98

3.2.1.3. Comparison of post-mortem and tuberculin test results	99
3.2.1.4. Bacteriology of tissues from cattle	101
3.2.2. Mycobacterium tuberculosis complex in human	103
3.2.2.1. Isolation of mycobacteria	103
3.2.2.2. Assessment of risk factors involved in the spread of mycobacteria infection	105
3.3. DISCUSSION	108
3.3.1. Bovine tuberculosis in cattle	108
3.3.2. Importance of <i>M. bovis</i> infection in man	117
3.3.3. Assessment of risk factors	119
3.3.4. Conclusion	123

CHAPTER 4: EVALUATION OF MOLECULAR TECHNIQUES FOR IDENTIFICATION AND TYPING OF MYCOBACTERIUM TUBERCULOSIS COMPLEX ORGANISMS

4.1. INTRODUCTION	125
4.1.1. The aims of the study	129
4.2.MATERIALS AND METHODS	131
4.2.1. Bacterial strains	131
4.3. RESULTS	131
4.3.2.PCR results	131
4.3.2.1. Preliminary PCR results	131
4.3.2.2. Optimisation of PCR	136
4.3.2.3. Specificity of PCR protocols	140
4.3.3. Molecular typing - IS986 RFLP	140
4.4. DISCUSSION	144

CHAPTER 5: DEVELOPMENT OF MOLECULAR BIOLOGY TECHNIQUES FOR TYPING AND DETECTION OF *M. BOVIS*

5.1. INTRODUCTION	149
5.1.1. Development of simplified molecular typing technique	149
5.1.2. An attempt to develop a <i>M. bovis</i> specific DNA probe.	150
5.1.3. Development of multiplex PCR able to distinguish species of the <i>M. tuberculosis</i> complex	152
5.1.4. Aims of the study	153
5.2.. RESULTS	154
5.2.1. Intersegment PCR	154
5.3.. RESULTS	158
5.3.1. Intersegment PCR	158
5.3.2. Development of <i>M. bovis</i> specific probe	169
5.3.2.1. Random Amplifying Polymorphic DNA (RAPD) PCR	169
5.3.2.2. Cloning of <i>f2b</i> and <i>f3b</i> RAPD PCR fragments	169
5.3.2.3. Sequencing of cloned <i>f2b</i> and <i>f3b</i> DNA fragments	171
5.3.2.4.. Sequence analysis	171
5.3.2.5. Specificity of the sequenced DNA for <i>f2b</i> and <i>f3b</i> to <i>M. bovis</i>	176
5.4. DISCUSSION	179
5.4.1. PCR fingerprinting	179
5.4.2 Development of <i>M. bovis</i> specific probe	183

CHAPTER 6: MOLECULAR EPIDEMIOLOGY OF THE *M. TUBERCULOSIS* COMPLEX ORGANISMS IN MAN AND CATTLE IN TANZANIA

6.1. INTRODUCTION	187
6.1.1. The aims of the study	192
6.2. MATERIAL AND METHODS	193
6.2.1. Mycobacterial isolates	193

6.2.2. Genomic DNA extraction	194
6.2.2. Molecular identification	194
6.2.3. Molecular typing	194
6.2.3.1. Restriction fragment length polymorphism	194
6.2.3.2. Spoligotyping	194
6.2.4. DNA fingerprint analysis	195
6.2.5. <i>M. bovis</i> genetic relatedness	195
6.3. RESULTS	196
6.3.1. Molecular identification	196
6.3.1.1. Multiplex IS986/mtp40 PCR	196
6.3.1.2. <i>M. bovis</i> specific PCR	196
6.3.2. Molecular typing	199
6.3.2.1. RFLP analysis of <i>M. bovis</i> strains using pTBN12 as a genetic marker	199
6.3.2.2. RFLP analysis of the <i>M. tuberculosis</i> complex strains using IS986 as a genetic marker	199
6.3.2.3. DNA polymorphism of strains of <i>M. tuberculosis</i> and <i>M. bovis</i> as determined by spoligotyping technique	209
6.3.2.4. Comparison of RFLP and spoligotype	214
6.3.3. The genetic relatedness of strains of <i>M. bovis</i> isolated in Tanzania	216
6.4. DISCUSSION	218
6.4.1. Molecular identification	218
6.4.2. Molecular typing	222

CHAPTER 7: THE DEVELOPMENT OF A MODIFIED LIQUID MEDIUM FOR THE GROWTH OF *MYCOBACTERIUM BOVIS* FOR MOLECULAR METHODS

7.1. INTRODUCTION	230
7.1.1. Aims of the study	232
7.2. MATERIALS AND METHODS	232

7.2.1. Mycobacterium strains	232
7.2.2. Media preparation	233
7.2.3. Experimental design	234
7.2.3.1. Experiment 1. Evaluation of the effect of sodium pyruvate	234
7.2.3.1.1. Viable counts	234
7.2.3.1.2. Wet weights	234
7.2.3.2. Experiment 2. Determination of growth rate	235
7.2.4. Statistical analysis	236
7.3. RESULTS	236
7.3.1. Experiment 1. Effect of sodium pyruvate	236
7.3.2. Experiment 2. Determination of rate of growth	241
7.4. DISCUSSION	243

CHAPTER 8: GENERAL DISCUSSION

8.1. INTRODUCTION	248
8.2. GENERAL EPIDEMIOLOGY OF BOVINE TUBERCULOSIS IN TANZANIA	250
8.3. MOLECULAR EPIDEMIOLOGY	256
8.4. CONCLUDING REMARKS	262

REFERENCES	266
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APPENDICES	xiii
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LIST OF FIGURES

Figure no.	Figure title	Page no.
1.1	The status of tuberculosis in cattle in Africa and the Middle East 1986-1990	30
1.2	Map of Tanzania indicating project areas and locations of VICs	48
1.3	The trend of tuberculosis in man in Tanzania (1983-1991)	51
1.4	The relationship of cattle keeping and proportion of cases of extra-pulmonary tuberculosis to total tuberculosis cases (1990)	52
2.1	The map of the Southern Highlands of Tanzania showing the location of slabs	57
2.2	Primers for various PCRs- primer sequence, position and size of product	75
2.3a	The physical map of IS986 (1.35Kb)	77
2.3b	The physical map of pTBN12 (3.8Kb)	77
2.4	A stylised diagram showing the principle of spoligotyping	83
3.1	The relative reactivity of cattle to bovine and avian PPDs	92
3.2	The effect of herd size on the incidence of bovine tuberculosis in herds and cattle	94
3.3	The reactivity of 224 lesion positive cattle with completed tuberculin test	101
4.1	The polymerase chain reaction	127
4.2	Polyacrylamide gel showing amplification products of <i>M. tuberculosis</i> and <i>M. bovis</i> strains from IS986 PCR	132
4.3	Polyacrylamide gel showing amplification products of <i>M. tuberculosis</i> and <i>M. bovis</i> strains from IS1081 PCR	133
4.4	Polyacrylamide gel showing amplification products of <i>M. tuberculosis</i> and <i>M. bovis</i> strains from MPB70 PCR	134

4.5	Polyacrylamide gel showing amplification products of <i>M. tuberculosis</i> strains from <i>mtp40</i> PCR	135
4.6	Polyacrylamide gel showing the effect of different concentrations of template DNA on IS986 PCR	137
4.7	Polyacrylamide gel showing the effect of different concentrations of template DNA on IS1081 PCR	138
4.8	Polyacrylamide gel showing the effect of different concentrations of template DNA on by MPB70 PCR.	139
4.9	IS986 RFLP DNA fingerprint analysis of 10 strains of <i>M. tuberculosis</i>	142
4.10	IS986 RFLP DNA fingerprint analysis of 17 strains of <i>M. bovis</i> and <i>M. tuberculosis</i>	143
5.1	The different permutations of intersegment PCR	156
5.2	Example of DNA fingerprinting by intersegment amplification	157
5.3	IS986↔IS986 (primers G4211/B976) intersegment PCR patterns	160
5.4	IS1081↔IS1081 (primers G4208/G4210) intersegment PCR patterns	161
5.5	IS986↔IS1081 (primers G4211/G4210) intersegment PCR patterns	162
5.6	IS986↔MPB70 (primers G4211/G4209) intersegment PCR patterns	163
5.7	IS1081↔MPB70 (primers G4211/ G4209) intersegment PCR patterns	164
5.8	IS986↔ <i>mtp40</i> (primers G4211/G4207) intersegment PCR patterns	165
5.9	IS1081↔ <i>mtp40</i> (primers G4208/G4207) intersegment PCR patterns	166
5.10a&b	Sensitivity of intersegment PCR on a high concentration of template DNA (100ng)	167

5.11	IS986↔IS986 (primers G4211/B976) intersegment PCR patterns produced by strains of atypical mycobacteria	168
5.12	Agarose gel showing amplimers of RAPD PCR for strains of <i>M. bovis</i> and <i>M. tuberculosis</i> indicating the fragments <i>f2b</i> and <i>f3b</i>	170
5.13	The nucleotide sequence and deduced amino acid sequence of <i>f2b</i>	173
5.14a	The nucleotide sequence and deduced amino acid sequence of <i>f3b</i>	174
5.14b	Peptide sequence match between <i>f3b</i> , <i>M. tuberculosis</i> cosmid and <i>rfbE</i> gene of <i>Yersinia enterocolitica</i>	175
5.15a&b	Southern blot hybridisation of genomic DNAs of <i>M. tuberculosis</i> , <i>M. bovis</i> and <i>M. avium</i> with a) <i>f2b</i> and b) <i>f3b</i> RAPD PCR products as probes	177
5.16	Southern blot hybridisation with a DNA probe obtained from <i>BglIII/EcoRI</i> digestion of the <i>f3b</i> from cloned DNA.	178
6.1	The pTBN12 RFLP fingerprint patterns for strains of <i>M. bovis</i> from cattle and man.	200
6.2	The pTBN12 RFLP types for strains of <i>M. bovis</i> from cattle and man	201
6.3	The geographical distribution of the pTBN12 RFLP types for strains of <i>M. bovis</i>	202
6.4	The IS986 RFLP fingerprint patterns for the strains of the <i>M. tuberculosis</i> complex from man.	205
6.5	The distribution of IS986 RFLP types for isolates of the <i>M. tuberculosis</i> complex from cattle and man	206
6.6	The geographical distribution of IS986 RFLP types for strains of <i>M. tuberculosis</i> complex.	207
6.7	The distribution of the IS986 RFLP types among strains of the <i>M. tuberculosis</i> complex	208
6.8	The spoligotype patterns for the strains of the <i>M. tuberculosis</i> complex from man and cattle	210

6.9	The distribution of spoligotypes for isolates of the <i>M. tuberculosis</i> complex from cattle and man	211
6.10	The geographical distribution of spoligotypes types for strains of the <i>M. tuberculosis</i> complex	212
6.11	The distribution of the spoligotypes among strains of the <i>M. tuberculosis</i> complex	213
6.12	The comparison of RFLP types to corresponding spoligotypes in strains of the <i>M. tuberculosis</i> complex	215
7.1	The comparison of viable counts produced by <i>M. bovis</i> and <i>M. tuberculosis</i> on Middlebrook 7H9 liquid medium with and without pyruvate	238
7.2	Comparison of growth of <i>M. bovis</i> and <i>M. tuberculosis</i> in Middlebrook 7H9 broth with and without pyruvate	239
7.3	The comparison of the mean wet weights produced by strains of <i>M. bovis</i> and <i>M. tuberculosis</i> grown on Middlebrook 7H9 broth with and without pyruvate	240
7.4	Comparison of mean viable counts and mean wet weight of the strains of <i>M.bovis</i> and <i>M. tuberculosis</i> harvested at the end of weeks 4, 5 and 6.	240
7.5	The wet weight (mg/ml) yields obtained from cultivation of <i>M. bovis</i> in different volumes of modified Middlebrook 7H9 broth	242

LIST OF TABLES

Table no.	Table title	Page no.
1.1	Classification of tubercle bacilli according to their epidemiological importance	6
1.2	The approved species of mycobacteria and the newly identified species	7
1.3	Differentiation of strains of the <i>M. tuberculosis</i> complex	10
1.4	Comparison of the molecular weight and base ratio of DNAs of species of the <i>M. tuberculosis</i> complex and selected MOTTs	12
1.5	DNA elements for detection of the <i>M. tuberculosis</i> complex	14
1.6	Comparison of the detection rates of mycobacteria from clinical specimens by PCR and conventional techniques	15
1.7	Results of studies on sensitivity of single comparative intradermal tuberculin test	23
1.8	Results of studies on sensitivity and specificity of ELISA in detecting tuberculous cattle	25
1.9	Reported cases of tuberculosis due to <i>M. bovis</i> in animal species other than cattle	32
1.10	A list of publications of the isolation of <i>M. bovis</i> from human cases of tuberculosis	38
1.11	Organs condemned due to bovine tuberculosis in Mbarali Sub-District for the year 1991	46
1.12	Results of Zonal tuberculin testing in Tanzania (1975 - 1990)	49
3.1	Validation of performance of tuberculin test in the Usangu Plains	91
3.2	Distribution of reactor cattle according to geographical location	92
3.3	Distribution of reactor herds according to geographical location	94

3.4	The distribution of reactors cattle tested in the Mid-Usangu area by village of origin	95
3.5	Results of tuberculin testing of cattle at Matebete Village	96
3.6	Comparison of the effect of various cattle variables on tuberculin reactivity of cattle in the Usangu Plains	97
3.7	The distribution of cattle with visible lesions (VL) at different stations	98
3.8	Distribution of tuberculous lesions in lesion positive cattle	99
3.9	The ability of tuberculin test to detect lesion positive at routine and enhanced post-mortem	100
3.10	Isolation and preliminary identification of mycobacteria from cattle specimens by SUA laboratory	102
3.11	Identification of species of mycobacteria from cattle specimens by SMRL	103
3.12	The isolation rate of mycobacteria from human cases of tuberculosis in Tanzania	104
3.13	The distribution of <i>Mycobacterium</i> species amongst the positive cases of tuberculosis	105
3.14	Analysis of risk factors associated with cases of tuberculosis in man	107
4.1	The results of amplification of 49 strains of mycobacteria using four different PCR protocols	140
5.1	The PCR typing techniques for the <i>M. tuberculosis</i> complex	150
5.2	Primer sequences for intersegment PCR amplifications	155
5.3	Primers used to sequence cloned <i>f2b</i> and <i>f3b</i> RAPD PCR fragments	171
6.1	Selected recent epidemiological studies which have utilised molecular techniques to trace the source or the index case responsible for the outbreak or establish a clonal relationship of prevailing strains of <i>M. tuberculosis</i>	190

6.2	Application of molecular typing to studies of the epidemiology of <i>M. bovis</i> infection in animals and man	191
6.3	Summary of results comparing identification and typing of the <i>M. tuberculosis</i> complex strains by standard biochemical tests and molecular methods.	197
6.4	The categorisation of strains of the <i>M. tuberculosis</i> complex according to different IS986 RFLP clusters	204
6.5a	The genetic relatedness value (GRV) amongst pTBN12 RFLP types of <i>M. bovis</i> isolates from Tanzania	217
6.5b	The genetic relatedness values (GRV) amongst spoligotypes of <i>M. bovis</i> isolates from Tanzania	217
7.1	The mean viable counts at the end of week four post inoculation	241
7.2	The rate of growth of <i>M. bovis</i> on different volumes of the modified Middlebrook 7H9 broth	242

ABBREVIATIONS

χ^2	chi square
ADC	albumin dextrose enrichment supplement
AFB	acid-fast-bacilli
AIDS	acquired immuno-deficiency syndrome
AMTD®	“amplified <i>Mycobacterium tuberculosis</i> direct test”
AP-PCR	arbitrarily primed PCR
BCG	bacilli-Calmette-Guerin
bp	base pair(s)
BTB	Bovine Tuberculosis Project
cfu	colony forming unit
CMI	cell mediated immune responses
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine phosphate
df	degrees of freedom
dGTP	deoxyguanosine triphosphate
DIG	digoxigenin
DNA	deoxyribonucleic acids
dNTP's	deoxynucleotide phosphates
DR	“Direct Repeat”
DTLC	the District Tuberculosis and Leprosy Co-ordinator
dTTP	deoxythymidine triphosphate
EA/BMRC	East African and British Medical Research Council
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immuno-sorbent assay
EP	extra-pulmonary
EVRET-94	the Edinburgh Veterinary Research Expedition Team -1994
FAO	Food and Agriculture Organisation
G-C	guanosine-ctyosine
GE	Guanidine isothiocyanate
HIV	human immuno-deficiency virus

HSP	heat shock protein
IFN γ	interferon-gamma
IgG	immunoglobulin Class G
IPTG	isopropyl- β -D-thiogalactopyranoside
IS	insertion sequence
IUATLD	International Union Against Tuberculosis and Lung diseases
IUT	International Union for Tuberculosis
Kb	kilo base (s)
kDa	kilo Dalton
L-B broth	Luria-Bertani broth
L-J	Loewenstein Jensen
LMP	low melting point
ln	lymph node
lnn	lymph nodes
LTT	lymphocyte transformation test
MMC	Muhimbili Medical Centre
MOTTs	mycobacteria other than tuberculosis
MPB64	mycobacterial protein bovis 64
MPB70	mycobacterial protein bovis 70
MRI	Moredun Research Institute
M.tb	<i>M. tuberculosis</i>
MTBC	the <i>M. tuberculosis</i> complex
mtp40	mycobacterium tuberculosis protein 40
MTPR	major polymorphic tandem repeats
NTLP	National Tuberculosis and Leprosy Programme (Tanzania)
NVL	non-visible-lesion
OIE	Office International des Epizooties
p	probabilty value
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
PGRS	polymorphic G-C rich sequence

pMBA2	plasmid <i>M. bovis</i> A2
pMTb4	plasmid <i>M. tuberculosis</i> 4
PNB	para-nitro benzoic acid
PPD	purified protein derivatives
pTB233	plasmid tuberculosis 233
pTBN12	plasmid <i>M. tuberculosis</i> number 12
r	correlation coefficient
RAPD	random amplified polymorphic DNA
REA	restriction enzyme analysis (REA)
RFLP	restriction fragment length polymorphism
RR	risk ratio
rRNA	ribosomal RNA
SCIT	single intradermal comparative test
SDS	sodium dodecyl sulphate
SHZ	Short Horn Zebu
SIT	single intradermal test
SMRL	Scottish <i>Mycobacterium</i> Reference Laboratory
SSC	sodium chloride and sodium citrate buffer
SSPE	sodium chloride, sodium dihydrophosphate and EDTA buffer
SUA	Sokoine University of Agriculture, Morogoro, Tanzania
T/BMRC	Tanzania/British Medical Research Council
TB	tuberculosis
TBE	tris-HCl, boric acid and EDTA
TCH	thiophene-2-carboxylic acid hydrazide
TEMED	N,N,N',N' - tetramethylethylenediamine
UV	ultra violet
VICs	Veterinary Investigation Centres
VL	visible-lesion
v/v	volume by volume
v/w	volume by weight
WHO	World Health Organisation

X-gal	5-bromo-4-chloro-indolyl- β -D-thiogalactopyranoside
ZN	Ziehl-Neelsen

CHAPTER 1

INTRODUCTION

CHAPTER 1. INTRODUCTION

1.1. REVIEW OF THE LITERATURE

1.1.1. HISTORY OF TUBERCULOSIS

The history of tuberculosis has been reviewed extensively by Cave (1939), Grange (1982;1988), Bloom and Murray (1992), Bates (1993) and Bloom (1994). These authors have provided chronological information describing the disease caused by tubercle bacilli. The documentation of the first disease conditions caused by tubercle bacilli of bovine type i.e. tuberculous spondylitis (or Pott's disease) existed in dynastic Egypt at around 3700 BC (Zimmerman 1977). As indicated by Bate and Stead (1993), *Mycobacterium tuberculosis* might have evolved later from the mutation of *M. bovis*, as the conditions of pulmonary tuberculosis described by Hippocrates as phthisis, which is derived from Greek word "wasting away", appeared in literature around 5 BC. It can be speculated that the evolution of *M. tuberculosis* from *M. bovis* and its success as a human pathogen might have arisen from some environmental condition existing in human beings but not in cattle during evolution (Scorpio and Zhang 1996). It is also speculated that man might have acquired *M. tuberculosis* from sub-human primates who are equally susceptible to *M. tuberculosis* and were known to be infected with tuberculosis prior to man (Daniel *et al.*, 1994).

The early civilisation and inter-community migrations enabled the early spread of the disease in various parts of the world. For example, in the 18th to early 19th century, tuberculosis prevalence peaked in Western Europe and as the Western Europeans moved around the world, they left behind a trail of tuberculosis epidemics (Daniel *et al.*, 1994). For example, in Latin and South America the hispanic invasion of the continent resulted in the introduction of tuberculosis. However, recent DNA technology has indicated the presence of a DNA element, specific for *M. tuberculosis*, in pre-hispanic Peruvian mummies several thousand years old (Holmes 1994). In Africa, particularly Sub-Saharan Africa, the disease appeared to be unknown until as late as the early 20th century, when this region was colonised

(Cummins 1920). The reason for the absence of the disease has been attributed to the social structure that existed at that time, when Africans lived in small remote villages where conditions for airborne transmission of the tubercle bacillus were unfavourable (Cummins 1920; Lichenstein 1928).

The causative agent(s) of tuberculosis remained unknown for a considerable time, despite the early reports indicating its infectious nature. Experimental work on “a consumptive” carried out by Jean-Antoine Villemin in 1868 demonstrated that sputum and caseous material from cattle and man produced tuberculosis in rabbits, and moreover, material from cattle produced severe lesions in rabbits compared to analogous material from man (Collins and Grange 1983). Similarly, the indications of bovine tuberculosis as a zoonosis came to light at the beginning of the 19th century. Carmichael in 1810 and Kleckle in 1846, observed that tuberculous lymphadenitis (scrofula) was more common among infants given cow’s milk than among those that were breast fed and thus incriminated cow’s milk as the source of this disease (Collins and Grange 1983). Furthermore, the danger of eating meat from tuberculous cattle is depicted in the Mosaic laws (Leviticus 22:27), when the disease was called ‘wen’ or ‘scurvy’ of cattle (Collins and Grange 1983).

On March 24, 1882, Robert Koch made a presentation to the Berlin Physiological Society and announced that he had observed and cultured the bacillus responsible for the disease “Tuberkelbacillen” (tubercle bacillus) (Grange and Bishop 1982). Zopf in the following year proposed the name “*Bacterium tuberculosis*” (Collins and Grange 1983). The generic name ‘*Mycobacterium*’ was introduced by Lehman and Neumann in 1896, meaning fungus-bacterium, due to fungus like pelicles produced by acid - fast bacilli when cultured on liquid medium (Grange 1982). Therefore, the full title for tubercle bacillus discovered by Koch was ‘*Mycobacterium tuberculosis* (Zopf) Lehmann and Neumann’ (Grange 1982).

Although the work of Theobald Smith (1898) and that of Antonie Villemin, some 30 years earlier, showed that the tubercle bacillus, which affected cattle, to be different from that affecting man, but caused similar disease in man as human tubercle bacillus. Robert Koch disregarded those findings, and in 1901, while addressing the British Congress on Tuberculosis, revealed his reservations regarding

the existence of a tubercle bacillus specific to cattle causing disease in man, by informing the audience that “human subjects are immune against infection with bovine bacilli or is so slightly susceptible that it is not necessary to take any steps to counteract risk of infection (Francis 1959). This submission produced a consternation amongst the veterinary/medical professions, and prompted the formation of the Royal Commission whose task was mainly to investigate the role of bovine bacilli as cause of disease in man (Francis 1959). The final conclusion reached by the Royal Commission in 1911 was that man is equally susceptible to bovine tubercle bacilli and should be added to the list of animals affected by the bovine tubercle bacillus (Francis 1959).

Another sequel to Koch’s *ex cathedra* pronouncement which paved the way to the development of a vaccine for the control of human tuberculosis, was the firm submission that bovine and human type bacilli would not produce reciprocal disease in man and cattle respectively. This view led Koch and his associate to prepare a vaccine for control of bovine tuberculosis from a human tubercle bacillus, supposedly attenuated by ageing. The vaccine made by Koch termed ‘Tauruman’ and a similar version of it produced by another Germany scientist, von Behring, achieved some popularity until 1913 when Griffith demonstrated that the vaccinated cattle excreted viable and virulent human tubercle bacillus in their milk (Collins and Grange 1983). However, it is believed that French scientists, Calmette and Guerin were inspired with Koch’s research output and they passaged a strain of bovine type tubercle bacillus by growing it on bile medium for some 300 generations. Over a period of twelve to thirteen years, the organism was found to have lost its power to produce tuberculosis, but had the ability to establish itself in susceptible animals without actually producing the disease (Larson and Evans 1929). The vaccine named after the founders, Bacilli-Calmette-Guerin (BCG) is up to the present day, being used to protect the human population against tuberculosis.

1.1.2. NOMENCLATURE

The generic name *Mycobacterium* was introduced by Lehmann and Neumann in the first edition of the Atlas of Bacteriology published in German in 1896. At that time, the genus contained only two species, namely; *Mycobacterium tuberculosis* and *Mycobacterium leprae*. *Mycobacterium tuberculosis* represented both the tubercle bacilli isolated from cattle and that from man. Theobald Smith (1898) observed differences between the two strains in cultural growth and virulence for guinea pigs, rabbits and cattle which suggested they were separate entities. Hence, the fourth edition of Lehmann and Neumann's Atlas of Bacteriology (1907) classified the two strains as distinct species although closely related and were termed *Mycobacterium tuberculosis typhus humanus* and *Mycobacterium tuberculosis typhus bovinus* respectively (Grange 1982). The 4th edition of Bergey's Manual of Determinative Bacteriology re-designated the two strains *Mycobacterium tuberculosis var hominis* and *Mycobacterium tuberculosis var bovis* (Grange 1982). The name *Mycobacterium bovis* for the bovine strain was first published in 1970 by Karlson and Lessel.

In 1936, Wells isolated a strain of *Mycobacterium* that was causing tuberculous lesions in voles (*Microtus agrestis*) and which was later termed as a "third mammalian type of tubercle bacilli". Brooke (1941) termed the organism *Mycobacterium tuberculosis var muris*, Smith *et al.* (1948) gave this species a specific name *Mycobacterium muris*, but as this name had already been applied to an unrelated organism it was changed to *Mycobacterium microti* in the 7th Edition of Bergey's Manual of Determinative Bacteriology (Reed 1957). This strain has properties which are between those of the human and bovine tubercle bacilli. However, it was found to exert an insignificant effect on human health and thus has been used as a vaccine against human tuberculosis (Wells and Wylie 1959). The species *Mycobacterium africanum* was first described by Castets *et al.* (1969). This species was recognised among the group of strains causing tuberculosis in tropical Africa and having properties intermediate between the human and bovine types. This group is further subdivided into two sub-groups, *Mycobacterium africanum* I which was isolated from people living in the West African Equatorial Region and

Mycobacterium africanum II which was isolated from human cases residing in Rwanda and Burundi (Castets 1969). Some biochemical properties of these strains resemble those of *M. tuberculosis* and *M. bovis* respectively (Table 1.1).

The resemblance of these strains of *M. africanum* to *M. tuberculosis* and *M. bovis* casts doubts on their designation as separate species (Prat *et al.*, 1974). These workers, together with Ratledge and Stanford (1982) and Grange (1982), have emphatically opposed the classification put forward in the list of approved species of mycobacteria by Skerman *et al.* (1980) and suggest that *M. bovis*, *M. africanum* and *M. microti* should be regarded as subspecies of *M. tuberculosis*.

Although the recent Bergey's Manual of Determinative Bacteriology (9th Edition - 1994) has classified mycobacteria according to their growth characteristics into two main groups, slow growing species of *Mycobacterium* and rapidly growing species (and subspecies) of *Mycobacterium*, the Skerman list of approved names for mycobacteria provides a rather comprehensive list of species of this genus (Table 1.2; Skerman *et al.*, 1980). The newly identified species are not included in the above two lists (Table 1.2).

Table 1.1. Classification of tubercle bacilli according to their epidemiological importance (Collins *et al.*, 1983)

Species	Biochemical characteristics			
	Oxygen preference	TCH sensitivity	Nitratase activity	Pyrazinamide sensitivity
<i>M. bovis</i>	M	-	-	R
<i>M. bovis</i> BCG	A	-	-	R
<i>M. africanum</i> I	M	-	-	S
<i>M. africanum</i> II	M	-	+	S
<i>M. tuberculosis</i> (asian type)	A	-	+	S
<i>M. tuberculosis</i> (classical)	A	+	+	S

A , aerobic; M, microaerophilic; R, resistant; S, sensitive; TCH, thiophene-2-carboxylic acid hydrazide; +, positive; -, negative.

Table 1.2. The approved species of mycobacteria according to Skerman *et al.* (1980), and the newly identified species (marked with asterisks).

<i>M. africanum</i>	<i>M. fallax</i> *	<i>M. malmoense</i>	<i>M. simiae</i>
<i>M. agri</i> *	<i>M. flavescens</i>	<i>M. marinum</i>	<i>M. smegmatis</i>
<i>M. aichiense</i> *	<i>M. fortuitum</i>	<i>M. microti</i>	<i>M. sphagni</i> *
<i>M. asiaticum</i>	<i>M. gadium</i>	<i>M. morioakaense</i> *	<i>M. szulgai</i>
<i>M. aurum</i>	<i>M. gastri</i>	<i>M. neoaurum</i> *	<i>M. terrae</i>
<i>M. austroafricanum</i> *	<i>M. genavense</i> ****	<i>M. nonchromogenicum</i>	<i>M. thermoresistibile</i>
<i>M. avium</i>	<i>M. gilvum</i>	<i>M. obuense</i> *	<i>M. tokaiense</i> *
<i>M. bovis</i>	<i>M. gordonae</i>	<i>M. parafortuitum</i>	<i>M. triviale</i>
<i>M. celatum</i> **	<i>M. haemophilum</i>	<i>M. paratuberculosis</i> ^a	<i>M. tuberculosis</i>
<i>M. chelonae</i>	<i>M. interjectum</i> ***	<i>M. phlei</i>	<i>M. ulcerans</i>
<i>M. chitae</i>	<i>M. intracellulare</i>	<i>M. porcinum</i> *	<i>M. vaccae</i>
<i>M. chubuense</i> *	<i>M. kansasii</i>	<i>M. poriferae</i> *	<i>M. xenopi</i>
<i>M. conspicuum</i> ***	<i>M. komossense</i>	<i>M. pulveris</i> *	
<i>M. cookii</i> *	<i>M. lentiflavum</i> ***	<i>M. rhodesiae</i> *	
<i>M. diernhoferi</i> *	<i>M. leprae</i>	<i>M. scrofulaceum</i>	
<i>M. duvalii</i>	<i>M. lepraemurium</i>	<i>M. senegalense</i>	

* Bergey's Manual of Determinative Bacteriology 9th Edition

** Butler *et al.* (1994) *** Springer *et al.* (1993/1995/1996)

**** Bottiger *et al.* (1993)

^a Thorel *et al.* (1990) has reclassify this species as subspecies of *M. avium*

1.1.3. BACTERIOLOGY

A classical description of the organisms of *Mycobacterium tuberculosis* complex is that they are fastidious, slowly growing, strictly aerobic or micro-aerophilic, lipid rich, hydrophobic, acid fast bacterial rods (Wayne 1994).

1.1.3.1. Microscopic morphology

The tubercle bacilli belonging to the *M. tuberculosis* complex are Gram-positive and acid-fast (Steenken *et al.*, 1934; Middlebrook *et al.*, 1947). They might be either slightly curved or straight rod-shaped although beaded forms are occasionally present. Their appearance in stains produces either a cording formation or non-oriented clumps depending on the age of the culture (Shinnick and Good 1994), or virulence (Middlebrook *et al.*, 1947). There is a slight difference in the cording

patterns between the human type and bovine type tubercle bacilli, with the latter showing less tight cords (Middlebrook *et al.*, 1947). The property of acid fastness is due to the ability of mycobacteria to resist decolourization by acid alcohol during the Gram staining procedure. This property was first described by Paul Ehrlich in 1882 and later by Ziehl and Neelsen and forms the basis of the current widely used Ziehl-Neelsen (ZN) staining technique (Grange 1988; Daniel *et al.*, 1994). According to Steenken *et al.* (1934) the degree of acid fastness increases with the age of the culture of tubercle bacilli, and that strains of *M. bovis* BCG and avirulent *M. tuberculosis* (H37R_a) have a much lower degree of acid fastness than virulent strains.

1.1.3.2. Macroscopic morphology

Colonies of the *M. tuberculosis* complex organisms can easily be categorised into those with a rough (R) or smooth (S) appearance on the surface of the solid medium (Vestal and Kubica 1966; Runyon 1970; Steenken *et al.*, 1934; Middlebrook *et al.*, 1947). Rough colonies appear dry and white or buff in colour. Upon examination with lower power magnification lenses, these colonies appear to be composed of filaments or pseudocords which pile up near the centre, often producing a dark central spot (Vestal and Kubica 1966). *M. bovis* produces rough colonies which tend to be more delicate in appearance, formed from small filaments (Vestal and Kubica 1966). Smooth colonies have been found to be generally circular with a raised central area that gradually flattens outwards, that can be lobed, irregular and undulated or completely smooth (Vestal and Kubica 1966). Middlebrook and others (1947), on the other hand, have linked colonial morphology with virulence of the strains. They observed that the virulent variants had a tendency to form a thin veil which spread uniformly on the entire surface of the medium and tended to climb over the surface of the container, while avirulent variants had a much less tendency to spread and heaped up in more or less discrete islands which did not coalesce. Yates and co-workers (1978), described the colonial morphology of strains of the *M. tuberculosis* complex on the basis of the amount of growth on glycerol-containing egg medium. *M. tuberculosis* produced a eugonic growth (i.e. heaped-up, bread crumb-like colonies) while *M. bovis* produced dysgonic growth (i.e. small, effuse colonies).

1.1.3.3. Growth requirements

There is a considerable variation in the temperature at which mycobacterial species can grow, and this is one of the criteria to differentiate species of this genus (Marks 1976; Grange 1988). The *M. tuberculosis* complex organisms grow optimally at 37°C, members of *M. avium intracellulare* complex will grow favourably at 37°C to 45°C, whereas most of the mycobacteria other than tuberculosis (MOTT) grow at temperatures ranging from 25°C to 45°C, hence classification of MOTT into psychrophiles (growth at 25-37°C), mesophiles (growth at 37°C) and thermophiles (growth at 37-42°C) (Marks 1976).

Although members of the *M. tuberculosis* complex group are generally aerobic, *M. bovis* is micro-aerophilic. The two species produce colonies at different levels on semi solid medium; *M. bovis* grows as a narrow band below the surface, while *M. tuberculosis* produces colonies on the surface of the medium (Marks 1976). An atmosphere with 5-10% carbon dioxide has been found to enhance the growth of mycobacteria (Grange 1990). Some of the MOTTs require light during incubation and produce pigment; these are called photochromic. Others, which produce pigment regardless of presence of light, are called scotochromes, and those which do not are termed non-chromogenicum (Marks 1976).

As indicated above, mycobacteria can be divided into two major groups on the basis of their speed of growth; i.e slow and fast growing mycobacteria. The former include species of the tuberculosis complex with a generation time of 12-18 hours (Drobniewski *et al.*, 1994) which require a minimum of 4-6 weeks for growth to appear on conventional media (i.e. Loewenstein-Jensen, Stonebrink etc.) (Marks 1976; Grange 1988). The majority of the MOTTs are fast growers and will produce colonies on the surface of conventional media within two weeks (Marks 1976). *M. leprae* is the only species of this genus that cannot be cultivated *in vitro* (Grange 1990).

1.1.3.4. Identification

The identification and typing of species of mycobacteria belonging to the *M. tuberculosis* complex has proven to be difficult due to a high degree of homogeneity

among the strains. From a medical point of view, tests have been devised to determine whether a strain is *M. tuberculosis* (the frequent isolate of man) or the species is *M. bovis* or its variant from the veterinary point of view. Grange (1982) has cautioned that, “the members of the tuberculosis complex group are so closely related and that they should really be considered variants of a single species as the difference between them is so small”. Collins *et al.* (1983) originally provided a scheme for differentiating the species of this group on the basis of a number of biochemical tests, namely oxygen preference, nitratase activity, susceptibility to pyrazinamide, and sensitivity to thiophen-2- carboxylic acid (TCH) (Table 1.1). A variation from this scheme has recently been documented by Hoffner and others (1993). In the latter typing scheme, they utilised four biochemical tests *viz.* niacin production, nitratase reduction, pyrazinamidase activity, sensitivity to TCH to classify members of the *M. tuberculosis* complex into five biovars (Table 1.3).

The typing scheme, described above, shows the close resemblance of strains belonging to the *M. tuberculosis* complex. The authors of this scheme have suggested that in a situation where there is a likelihood of transmission of mycobacteria between animals and man, the distinction between species should be considered, otherwise all species should be regarded as biovars of one species (Hoffner *et al.* 1993) .

Table 1.3. Differentiation of strains of the *M. tuberculosis* complex according to Hoffner *et al.* (1993).

Biovar	Biochemical test			
	TCH	Nitratase	Niacin	Pyrazinamidase
1 (classical <i>M. bovis</i>)	S	-	-	-
2 (<i>M. bovis</i> variant)	S	-	-	+
3 (<i>M. bovis</i> / <i>M.tb</i> var)	S	-	+	+
4 (<i>M. tb</i> variant)	S	+	+	+
5 (classical <i>M. tb</i>)	R	+	+	+

R=Resistant S=Susceptible +=Positive -=Negative

The other conventional typing techniques applied in identification of organisms of the *M. tuberculosis* complex include; phagotyping, antibiogram typing, serotyping and chromatography. These techniques have offered alternative tests but without any significant improvement (Saunders 1995). For example, phagotyping had problems of reproducibility, and its discriminatory power was very limited as there were only three phagotypes identified (Saunders 1995). Similarly, serogrouping of *M. tuberculosis* strains was difficult as unrelated strains fell into only one group (Jones and Kubica 1968) and, also, that ELISA assays, using A60 antigen, have proved to be non-specific (Watt *et al.*, 1993). Although antimicrobial sensitivity could reveal polymorphisms among unrelated strains, it has been noticed that, in certain circumstances, all strains could be susceptible to all antibiotics (Saunders 1995). Analysis by chromatographic techniques of mycobacterial lipids can give a more rapid identification of mycobacteria, however, these techniques have, so far, received little attention as they have failed to differentiate members of the *M. tuberculosis* complex (Luquin *et al.*, 1991).

1.1.4. MOLECULAR DETECTION, IDENTIFICATION AND TYPING

The growth of knowledge on molecular biology has opened up a number of new approaches to the characterisation of micro-organisms. Of particular value are certain techniques that give insight into genotypic properties and, thus, complement the widely used phenotypic characterisation of the organisms. Several kinds of analysis performed upon nucleic acids furnish information about the genotype; for instance, the analysis of the genomic molecular weight, of base composition of DNA, the study of chemical hybridisation between nucleic acids isolated from different organisms and sequencing of nucleic acid.

The work of Baess (1979) and Imaeda (1985) have provided genotyping information of species of the *M. tuberculosis* complex and some of the MOTTs (Table 1.4).

Due to the high level of homology amongst the strains of the *M. tuberculosis* complex (Table 1.4), conventional strain identification and typing systems have

failed to discriminate clearly species within the *M. tuberculosis* complex and secondly, cannot differentiate strains of the same species from unrelated sources.

Table 1.4. Comparison of the molecular weight and base ratio of DNAs of species of the *M. tuberculosis* complex and selected MOTTs (modified from Imaeda 1985).

Strain	Genome molecular weight X 10 ⁹	Guanine-ctyosine content (mol%) range	% relatedness with <i>M. tuberculosis</i> reference strain H37R _v
a) <i>M. tb.</i> complex			
<i>M. tuberculosis</i>	1.9-2.0	62.7-63.3	100
<i>M. bovis</i>	1.8-1.9	62.7-63.3	98-100
<i>M. bovis</i> BCG	1.8-2.3	62.5-63.3	98-100
<i>M. microti</i>	1.9	62.3-63.3	ND
<i>M. africanum</i>	2.0	62.7	100
b) MOTTs*			
<i>M. avium</i>	2.3	66.7	27
<i>M. asiaticum</i>	2.0	63.1	9
<i>M. kansasii</i>	2.2	63.3	32
<i>M. intracellulare</i>	2.0	65.7	48
<i>M. scrofulaceum</i>	2.4	65.8	22
<i>M. haemophilum</i>	1.8	61.5	17
<i>M. marinum</i>	2.5	63.1	11

* MOTTs of pathogenic significance

1.1.4.1. Molecular detection and identification.

1.1.4.1.1. Polymerase chain reaction

The advances in the recombinant DNA technology pave the way to the development of the polymerase chain reaction (PCR) (Saiki *et al.*, 1985, 1988). PCR is able to detect and identify mycobacteria directly in a clinical sample, with similar sensitivity to culture and in a matter of hours rather than weeks (Collins *et al.*, 1992). Like all DNA probe techniques, the PCR requires that the mycobacterial cells are first lysed in order to release DNA for amplification. Standard enzymatic DNA extraction from

mycobacterial cells may be used (Hartskeerl *et al.*, 1989). Alternatively, since PCR does not require high molecular weight native DNA, harsher procedures may be used to lyse cells (Buck *et al.*, 1992; Folgueira *et al.*, 1993). The PCR product may be detected directly by gel electrophoresis and ethidium bromide or silver nitrate staining of agarose and polyacrylamide gels respectively, or by hybridisation with a labelled DNA probe. Alternatively, a second 'nested' PCR may be used to detect small quantities of PCR product increasing both specificity and sensitivity (MacFadden *et al.*, 1990). The application of PCR to mycobacterial detection requires DNA sequence information for design and synthesis of primers homologous to target DNA elements. DNA elements specific to the genus *Mycobacterium* can be divided into two major groups, i) those which are repeated elements e.g. insertion sequences (Table 1.5a) and ii) genetic specific elements responsible for the phenotypic expression of the micro-organisms e.g. protein, resistance encoding DNA elements (Table 1.5b).

In principle, PCR is a simple and rapid test for the detection of mycobacteria. However, as with other tests, evaluation of its performance, reliability and reproducibility for detection of mycobacteria from clinical specimens is required, particularly in comparison with conventional methods. A number of workers have shown its superiority in comparison to microscopy and culture (conventional and BACTEC system), (Table 1.6).

The reason for such a superior rate of detection arises from the ability of PCR to detect as low as 5 mycobacterial cells, equivalent to 0.1 picogramms of DNA (Cousins *et al.*, 1991; Kolk *et al.*, 1992; Kox *et al.*, 1994), as opposed to culture which requires the presence of at least 10^2 viable cells in clinical specimens for visible growth to be evident (Yaeger *et al.* 1967) and 10^4 cells for a positive ZN smear (Yaeger *et al.*, 1967).

Table 1.5. DNA elements for detection of the *M. tuberculosis* complex **a)** mycobacterial repeated (transposable) DNA elements **b)** Non transposable genetic elements

a) repetitive DNA in the *M. tuberculosis* complex (modified from Small and van Embden 1994)

Element	Size (bp)	Host range	Copy no.	Reference
IS6110	1355	MTBC*	0-25	McAdam <i>et al.</i> 1990; Thiery <i>et al.</i> 1990; Hermans <i>et al.</i> 1991.
IS1081	1324	MTBC	5-7	Collins and Stephens, 1991; van Soolingen <i>et al.</i> 1992.
DR	36	<i>M. xenopi</i> MTBC	10-50	Groenen <i>et al.</i> 1993; Hermans <i>et al.</i> 1992.
MTPR	10	MTBC <i>M. gordonae</i> <i>M. kansasii</i> <i>M. gastri</i> <i>M. szulgae</i>	>100	Hermans <i>et al.</i> 1992
PGRS	30	MTBC <i>M. gordonae</i> <i>M. kansasii</i> <i>M. gastri</i> <i>M. szulgae</i>	>100	Ross and Dwyer 1993; Small and van Embden 1994

* MTBC = the *M. tuberculosis* complex

b) Non-repeated genetic elements

Genetic element	genus/species specificity	Reference
MPB70	the <i>M. tuberculosis</i> complex	Cousins <i>et al.</i> 1991
<i>mtp40</i>	<i>M. tuberculosis</i>	del Portillo <i>et al.</i> 1991
16S rRNA	<i>Mycobacterium</i> spp.	Boddinghaus <i>et al.</i> 1990
Antigen 85	“	Fauville-Dufaux <i>et al.</i> 1992
65 kDa HSP	“	Brisso-Noel <i>et al.</i> 1989
MPB64	“	Shankar <i>et al.</i> 1990
38 kDa antigen	“	Sjobring <i>et al.</i> 1990
P36	“	de Wit <i>et al.</i> 1990
32 kDa antigen	“	Soini <i>et al.</i> 1992
dna J gene	“	Takewaki <i>et al.</i> 1993

Table 1.6. Comparison of the detection rates of mycobacteria from clinical specimens by PCR and conventional techniques.

No. samples	Conventional methods		PCR method		Reference
	Smear +ve (%)	Culture +ve (%)	samples +ve (%)	target DNA	
26	3 (11.5)	23 (88.5)	24 (92.3)	P36	de Wit <i>et al.</i> 1990
117	15 (12.8)	17 (14.5)	31 (26.5)	MPB64	Manjunath <i>et al.</i> 1991
96	NA	74 (77.1)	88 (91.7)	pMTb4	Sritharan <i>et al.</i> 1991
94	46 (48.9)	44 (46.8)	52 (55.3)	IS6110	Eisenach <i>et al.</i> 1991
30	8 (26.7)	11 (36.7)	18 (60.0)	<i>mtp40</i>	del Portillo <i>et al.</i> 1991
227	37 (16.3)	45 (19.8)	79 (34.8)	IS986	Kolk <i>et al.</i> 1992
75	48 (64.0)	71 (94.7)	75 (100.0)	IS6110	Folguera <i>et al.</i> 1993
78	26 (33.3)	29 (37.2)	36 (46.2)	IS6110	Kocagoz <i>et al.</i> 1993
313	110 (35.1)	123 (39.3)	226 (72.2)	IS6110	Nolte <i>et al.</i> 1993
667	59 (8.8)	80 (12.0)	90 (13.5)	IS6110	Forbes <i>et al.</i> 1993
218	79 (36.2)	95 (43.6)	104 (47.7)	IS6110	Kox <i>et al.</i> 1994

Despite this acclaimed high specificity and sensitivity of PCR amplification, this technique has its own shortcomings. The two major problems are (i) false-positive reactions due to contamination with DNA fragments from the previous amplifications (amplicons) or from environmental organisms, and (ii) false-negative reactions caused by inhibitors that interfere with the PCR reaction (Hawkey *et al.*, 1994). Furthermore, reproducibility of the results utilising different PCR protocols is questionable, as narrated by Noordhoek and co-workers (1994). They carried out a blind study to determine the specificity and sensitivity of PCR protocols as performed by seven different laboratories. These laboratories were supplied with aliquots of same samples. The results of this study found the level of false-positives was on average 20% (range 3 to 77%). Regarding sensitivity, they also found disparity among the laboratories. These discrepancies could be attributed to a number of reasons but notably among them was the lack of good laboratory practices (Noordhoek *et al.*, 1996) and lack of separation of the areas for carrying out different

preparatory manipulations prior to actual PCR amplification on the heating block (Bell 1989; Coote 1990).

The recently developed DNA technologies apart from providing better tools for the detection of mycobacteria (i.e. DNA probes, PCR, etc.), also has provided techniques for the accurate identification not only the *M. tuberculosis* complex (Tables 1.5a&b), but also individual species within the complex, for example, *M. tuberculosis* specific PCR (del Portillo *et al.*, 1991), *M. bovis* specific PCRs (Cousins *et al.*, 1991; Rodriguez *et al.*, 1995), and multiplex PCRs for differentiating members of the group (Sinclair *et al.*, 1995; Liebarna *et al.*, 1996; del Portillo *et al.*, 1996). Detailed discussion of the ability of PCR to identify species of the *M. tuberculosis* complex is provided in Chapters 4 through 6.

1.1.4.1.2. DNA-DNA hybridization technique

Apart from PCR, mycobacterial DNA can be detected directly in clinical specimens by the use of DNA probes, which hybridise specific DNA sequences of mycobacterium.

DNA probes for the detection of mycobacterial DNA have received a wide use in specialist mycobacteriology laboratories in developed countries due to their advantages over conventional detection systems. DNA probes are able to differentiate the strains differing in only a single base substitution, hence, have been found to be useful in differentiating species of taxa such as the *M. tuberculosis* complex and, also, the *M. avium-intracellulare* complex (Patel *et al.*, 1989).

Mycobacterial DNA probes are now manufactured in the form of commercial kits. For example, Gen-Probe produce three kits (Gen-probe, Inc. San-Diego, California US) based on probes complementary to the DNA sequence of the 16srRNA gene; i) the *M. tuberculosis* complex, ii) the *M. avium -intracellulare* complex, and iii) *M. gordonae*, (Eisenach *et al.*, 1988). Syngene, Inc. San-Diego, California, USA, produce a non -isotopic DNA probe for the *M. tuberculosis* complex, called SNAP[®] (Ford *et al.*, 1993). Accuprobe (Gen-probe Inc. San-Diego, California, US) also produce a non-isotopic probe for detection of the *M. tuberculosis* complex (Ford *et al.*, 1993). There are two main disadvantages associated with the use of the genetic

probes (*viz.* Accuprobe, Gen-probe, and SNAP). First, the cross reactivity of the probe intended for identification of the *M. tuberculosis* complex to MOTTs, notably *M. terrae* and *M. celatum* (Ford *et al.*, 1993; Butler *et al.*, 1994). The main reason for this cross reactivity is that the target gene is also present in these species with only a single mis-match at the site for the commercial probe (Emler *et al.*, 1995). Second, these probes lack sensitivity and can require a minimum of 10⁶ organisms to give positive results and so are unsuitable for direct identification of organisms in clinical specimens (Watt *et al.*, 1993).

Modifications to incorporate PCR for the detection of the same DNA element targeted by DNA probes have been introduced, the Gen-probe “amplified *Mycobacterium tuberculosis* direct test” (AMTD[®]) (Abe *et al.*, 1993; Jonas *et al.*, 1993). In order to validate the performance of these probes, a number of workers have compared AMTD[®] with conventional detection techniques and PCR for IS986 and found that AMTD[®] and IS986 PCR provided more sensitive and specific results when dealing with different kinds of clinical specimens (Abe *et al.*, 1993; Miller *et al.*, 1994). Recently, a rapid PCR-based test for detection of *M. tuberculosis*, the Roche AMPLICOR[®] *Mycobacterium tuberculosis* test (AMPLICOR - MTB; Roche Diagnostics, Somerville, N.J.) has been developed in a kit form for commercial use. The molecular basis of AMPLICOR-MTB[®] is the amplification of 16S rRNA gene sequence (D’amato *et al.*, 1995). As with the other DNA probes, and the AMTD[®], the AMPLICOR[®] was also evaluated by comparing its performance with conventional detection techniques, and was found to be superior (Carpentier *et al.*, 1995; D’amato *et al.*, 1995; Schirm *et al.*, 1995).

1.1.4.2. Molecular typing

Typing of strains is important for the epidemiological control of diseases such as tuberculosis in both man and animals, as it allows cases to be linked and outbreaks to be traced with greater precision.

DNA fingerprinting methods recently developed include the restriction enzyme analysis (REA) (Collins *et al.*, 1985), restriction fragment length polymorphism (RFLP) typing which is now widely used (van Embden *et al.*, 1993) and pulsed field

gel electrophoresis (PFGE) (Zhang *et al.*, 1992). These techniques require extraction of DNA from cultures followed by cutting the extracted DNA at a specific base sequence by restriction endonucleases generating numerous small- or large-sized fragments. The fragments of different molecular sizes are then separated by agarose gel electrophoresis, stained with ethidium bromide and visualised by ultra violet light to reveal the banding pattern. Some workers have made use of this banding pattern to type strains of *M. bovis* (Collins *et al.*, 1986; de Lisle *et al.*, 1990). However, REA has proven to be cumbersome to perform and the interpretation of results is difficult due to the high number of bands generated by frequent cutting enzymes (Crawford 1993). Better results have been obtained with the technique which utilises restriction enzymes that cut infrequently to produce a smaller number of large-sized fragments - the pulsed field gel electrophoresis (PFGE). It is easier to interpret the resulting banding pattern with this technique (Zhang *et al.*, 1992).

The standard RFLP analysis involves detection of specific restriction fragments by hybridisation of the Southern blots with labelled DNA probes. There is a number of DNA probes used for typing strains of the *M. tuberculosis* complex (Tables 1.5a&b). However, IS6110 (IS986/IS987) has been used most widely (Tables 6.1&6.2) in typing strains of the *M. tuberculosis* complex. The other probes include; polymorphic G-C rich sequence (PGRS), such as pTBN12 (Ross and Dwyer 1993) or pMBA2 (Bigi *et al.*, 1995), and direct repeats (DR) (Small and van Embden 1994) have been used occasionally to supplement IS6110 RFLP typing. Recently, DNA amplification techniques have been introduced for faster typing and for rapid identification of strains of the *M. tuberculosis* complex (Small and van Embden 1994). These techniques include spoligotyping (van Soolingen *et al.*, 1995), mixed-linker PCR (Haas *et al.*, 1993), arbitrary priming- PCR (Palittapongarnpim *et al.*, 1993 a&b), among many. Detail review of the techniques used in typing of strains of the *M. tuberculosis* complex is provided later (Chapters 5 and 6).

1.1.5. BOVINE TUBERCULOSIS IN CATTLE

1.1.5.1. Immune responses

The understanding of immune responses in bovine tuberculosis has been an extrapolation of findings arising from studies of tuberculosis in man and laboratory animals (Pritchard 1988; Neill *et al.*, 1994).

Lenzini *et al.* (1977) presented a hypothetical immunological spectrum in human tuberculosis to explain clinical, histological, bacteriological and immunological findings. At one end of the spectrum are highly reactive patients, with strong cell mediated immune responses (CMI) having well localised lesions but without tubercle bacilli in sputum and with little or no circulating antibodies, whereas at the other end, are patients with poor CMI, having high levels of circulating antibodies accompanied with heavy bacterial loads. A similar situation has been reported in *M. bovis*-infected cattle (Lepper *et al.*, 1977; Hanna *et al.*, 1992). Using production of IFN γ and IgG, as indicative of cellular and humoral response respectively, Ritacco and co-workers (1991) found that localised lesions were associated with high IFN γ and low specific IgG levels, and that extensive lesions were associated with high IgG antibody titres with low IFN γ . However, immune responses might be affected by a number of factors, including; poor state of nutrition, stress due to transportation, stress due to pregnancy and the presence of other debilitating disease conditions (Pritchard 1988; Doherty *et al.*, 1995). These factors may contribute to anergy i.e. the lack of responsiveness to screening tests in the presence of the disease (Lepper *et al.*, 1977).

Although knowledge of mechanisms of immune responses in bovine tuberculosis has been an extension of what has been found in *M. tuberculosis* infection in man, it is now evident that there might be minor differences. Rook *et al.* (1986) and Flesch and Kaufmann (1987) have reported production of interferon-gamma (IFN γ) as the key endogenous cytokine that triggers antimycobacterial effects of murine macrophages. Experimentally, murine macrophages have been shown to inhibit the growth *M. tuberculosis* in the presence of IFN γ , a feature not experienced with human macrophages (Rook 1987/90; Collins 1990).

1.1.5.2. Pathology

The pathology and pathogenesis of tuberculosis in cattle has been extensively described by Stamp and Wilson (1946), Stamp (1948), Francis (1958) and Jubb and Kennedy (1970). A little more has been added since that time.

Tuberculosis spreads in the body in two stages, the primary complex and post-primary dissemination. The primary complex consists of the lesions at the port of entry and in the local lymph nodes. A lesion at the point of entry is common when infection is by inhalation and when the infection occurs via the oral route the resulting lesions are normally found in organs other than those associated with respiratory tract (Radostits *et al.*, 1994). The majority of bovine tuberculosis cases are acquired by inhalation of *M. bovis* by droplet nuclei and the primary complex is found usually in the lungs and associated lymph nodes (Corner *et al.*, 1990; Lepper and Pearson 1973). Lesions occur predominantly in the caudal lobes and often in the distal third, bronchial and/or mediastinal lymph nodes are also affected (Stamp 1948; McIlroy *et al.*, 1986). The size and distribution of the lesions within the lungs and affected lymph nodes vary with the stage of the disease (Stamp 1948; McIlroy *et al.*, 1986).

Alimentary infection is thought to be a result of ingestion of expectorata heavily laden with bacteria from lungs (Francis 1947; Neill *et al.*, 1988b). In calves fed tuberculous milk, the primary focus is in pharyngeal and/or mesenteric lymph nodes (Radostits *et al.*, 1994), whereas in congenital tuberculosis, the primary complex is in the liver and portal lymph nodes. This form of disease usually spreads rapidly, leading to generalised disease and, in most cases, death is imminent (Neill *et al.*, 1994).

Following the primary complex stage, post-primary dissemination ensues, which may take the form of acute miliary tuberculosis affecting other parts of the body, notably the liver, kidneys, udder, meninges and serous cavities (Corner *et al.*, 1994; Neill *et al.*, 1988a). Depending on the sites of localisation of infection, clinical signs vary but, because the disease is always progressive, there is constant underlying toxæmia which causes weakness, debility and eventual death of the animal (Radostits *et al.*, 1994). Skin tuberculosis - *alias* 'dermatitis nodosa' or 'acid fast lymphangitis', characterised by strings of small abscesses following the track of superficial

lymphatic vessels, has been observed in cattle affected with bovine tuberculosis (Karlson 1962).

1.1.5.3. Clinical manifestation

The general clinical manifestations for bovine tuberculosis in cattle are not very specific, and clinical signs depend on which organs are involved (Radostits *et al.*, 1994). Emaciation not associated with any other condition should arouse suspicion of tuberculosis. A capricious appetite and fluctuating temperature are also indicative of the disease. Silent coughing, occurring intermittently upon inducement by either squeezing of the pharynx or exercise, is an indication of pulmonary tuberculosis at an early stage, while in an advanced stage, dyspnoea is the main feature (Radostits *et al.*, 1994). Diarrhoea is not a common feature in cases of alimentary tract involvement. However, enlargement of retropharyngeal lymph nodes may cause dysphagia and difficult breathing. Enlarged superficial lymph nodes may be easily palpable, though it is a rare event.

Urogenital system involvement might give rise to recurrent abortions close to parturition. An aborted foetus may survive but die quickly due to generalised tuberculosis. In chronic cases, purulent material laden with many bacteria will be discharged through the external genitalia (Radostits *et al.*, 1994).

Tuberculous mastitis is of major importance because of the danger to public health, and spread of the disease to calves. Clinically, the udder (particularly the rear quarters) will be indurated accompanied with hypertrophy, that descends from the upper part towards the teat opening. The consistency of milk changes as the disease progresses; at a later stage of the disease milk contains fine floccules and eventually resembles clear amber liquid (Radostits *et al.*, 1994).

1.1.6. DIAGNOSIS

1.1.6.1. Clinical diagnosis

Detection of clinically diseased cattle relies on examination of cattle by auscultation, percussion, body temperature measurement and also palpation of mammary gland

and superficial lymphnodes. However, as explained above, clinical manifestations are not pathognomonic for tuberculosis only (Radostits *et al.*, 1994). Additional specific tests are required to assess tuberculosis status in cattle.

1.1.6.1.1. Tuberculin test and in vitro cellular assays

Diagnosis of tuberculosis in cattle since the last century has depended mainly on tuberculin testing, where cattle reacting to bovine/human PPD were declared diseased. The history and development of the tuberculin test have been the subject of many reviews (Francis 1947; Ritchie 1953; Edwards and Edwards 1960; Snider 1982; Monaghan *et al.*, 1994).

Tuberculin test commonly used nowadays, is applied intradermally. Attempts have been made to use intravenous (Larsen and Kopecky 1965) and subcutaneous routes (Parker 1929) with little success. There are two kinds of intradermal skin tests commonly used nowadays; single intradermal test (SIT) using bovine tuberculin and single intradermal comparative test (SCITT) using avian and bovine tuberculins (Ritchie 1953). The SIT test is applied by the intradermal injection of tuberculin into a skin-fold. In Europe the skin of the neck is preferred, while in Northern America, Australia and New Zealand the caudal fold is preferred (Monaghan *et al.*, 1994).

A variation of the SIT test is the so called Stormont test (Radostits *et al.*, 1994), which is performed similarly to the SIT. The test involves further injection of the same tuberculin at the same site 7 days later. An increase of 5mm or more, 24 hours after the second injection, is a positive result. This test, although it is regarded to increase sensitivity, it is cumbersome as it requires at least three visits to the same animal.

The main disadvantage of the SIT is the lack of specificity and detection of non-visible-lesion (NVL) reactors. These NVL arise from the lack of specificity of antigens found in mammalian PPD, as there are common antigens shared with MOTTs, and even other non-mycobacteria micro-organisms such as *Norcadia* (Fifis *et al.*, 1991)

The SCITT was used initially as a measure to improve the specificity of detecting truly diseased cattle although sensitisation due to human type bacilli, or vaccination

may not be resolved. In this test, avian PPD and bovine PPD are injected simultaneously into two separate sites on the same side of the neck, approximately 12 cm apart and one above the other, and the test is read 72 hours later (Radostits *et al.*, 1994). The dosage rate according to EU standards are 0.1ml equivalent to 2000 International Units for avian PPD and 0.1 ml of bovine PPD equivalent to 2000 Community Tuberculin Units (Monaghan *et al.*, 1994). As with the SIT, reaction to both avian and bovine tuberculin is observed and measured at 72 hours after the injection of tuberculins. Lesslie and Herbert (1975), have provided the best guidelines for interpretation of tuberculin test in cattle.

As with any other screening test, a need to validate the test before its application to the field at large is of paramount importance (O'Reilly 1995). In the validation of the tuberculin test two parameters need a critical assessment; i) the sensitivity of the test i.e. the ability of the test to detect truly diseased animals (or proportion of test positive over total diseased) and ii) the specificity of the test i.e. the ability of the test to exclude non-diseased animals (or proportion of test negative over total non-diseased). As indicated by Monaghan *et al.* (1994) and O'Reilly (1995), a biological test will never achieve a 100% situation in both sensitivity and specificity. Another parameter that needs to be considered is the predictive value of the test. This parameter is directly proportion to the prevalence of the disease in the population tested. Table 1.7 as adopted from Monaghan *et al.* (1994) summarises results of SCITT as performed by a number of workers; it can be deduced that the sensitivity of SCITT varies from 77 to 95%.

In any application of a screening test, there are shortcomings which may have implications for the intended purpose of the test. One of the shortcomings associated with use of tuberculin tests in control of bovine tuberculosis by a test-and-slaughter policy is the question of false negative results (i.e. animals testing negative while they are actually diseased). False negative results may arise from a number of factors including, the inability of the animal to be sensitised with PPD due to the presence of other debilitating infections (Lepper *et al.*, 1977), immuno-suppression during the post-partum period (Kerr *et al.*, 1946a; O'Reilly 1987), malnutrition (Pritchard *et al.*, 1975; Francis *et al.*, 1978; Andrews 1989 personal communications to

Table 1.7. Results of studies on sensitivity of single comparative intradermal tuberculin test (Monaghan *et al.*, 1994).

Total infected cattle	Reactors (%)	Doubtful (%)	Sensitivity (%)	Study reference
300	91		91	Kerr <i>et al.</i> (1946a)
201	68	14	82	Kerr <i>et al.</i> (1949)
82	63.4	24.3	87.7	Paterson <i>et al.</i> (1958)
151	40	37	77	de Jong <i>et al.</i> (1969)
58	62	29	91	Lesslie <i>et al.</i> (1975)
91	68.8	26.2	95	O'Reilly <i>et al.</i> (1975)
68	62	28	90	O'Reilly (1986)

Dr. L. O'Reilly, Veterinary Laboratories, Department of Agriculture Food and Fisheries, Dublin, Ireland; O'Reilly and Daborn 1995; Cook *et al.*, 1996), desensitisation due to a recent tuberculin test (Kerr *et al.*, 1946b), the application of insufficient antigens and/or malfunctioning of tuberculin syringes (Monaghan *et al.*, 1994), and the use of tuberculin of low or reduced potency (Haagsma *et al.*, 1982; O'Reilly 1986). The serious implication of false-negative results is that the eradication/control programme will never achieve its target as a number of diseased animals will remain in the herds and continue to spread the disease to susceptible ones. On the other hand, false positives, mostly due to the presence of atypical mycobacterium (Karlson 1962) will result in the slaughter of many disease-free cattle.

In order to overcome the logistical problems associated with the use of tuberculin tests, several workers have developed laboratory-based tests able to measure cell mediated responses in similar manner to the tuberculin test. These include; the lymphocyte transformation (LTT) assay (Outteridge and Lepper 1973; Thoen *et al.*, 1980) and the gamma interferon (IFN γ) assay (Wood *et al.*, 1990; Rothel *et al.*, 1990). The LTT had a very limited use as it requires a laboratory able to handle radioactive materials. Although the IFN γ assay has been found to be 96.3% and 93.6% specificity and sensitivity respectively, as opposed to a sensitivity of 65.6%

and specificity of 98.8% produced by tuberculin test (Wood *et al.*, 1991), its use as a field test has been restricted to only a few countries (New Zealand, Australia and Ireland). This limited usage has been attributed to the high cost of the kit (Prof J.D. Collins/Dr. M. Monaghan, Department of Large Animal Clinical Medicine, University College Dublin, personal communications -1993/96).

1.1.6.1.2. Serological tests

Several tests able to detect humoral responses as a result of *M. bovis* infection in cattle have been developed and tested in the field with variable success. These tests include; enzyme linked immuno-sorbent assay (Table 1.8), bentonite flocculation test (Wallace *et al.*, 1968; Lepper *et al.*, 1973), indirect fluorescent antibody test (Lepper and Pearson 1975) and glutaraldehyde test (de Kantor 1993). Among the above mentioned tests, it is the enzyme linked immuno-sorbent assay (ELISA) which has been widely utilised in detection of tuberculosis in cattle. The summary of the specificity and sensitivity of ELISA as utilised by various groups shows that, various antigens can produce varying specificity and sensitivity (Table1. 8).

Table 1.8. Results of studies on sensitivity and specificity of ELISA in detecting tuberculous cattle

Study reference	antigen used	Sensitivity (%)	Specificity (%)
Thoen <i>et al.</i> 1984	bovine PPD	50	58.2
Ayanwale 1987	bovine PPD	98	65.6
Ritacco <i>et al.</i> 1987	bovine PPD	90	89.8
Auer 1987	crude antigen	88.7	52.6
Woods <i>et al.</i> 1992	MPB70	18.1	96.4
Wood and Rothel 1994	MPB70	49.5	98.2

1.1.6.2. Post-mortem diagnosis

A tentative diagnosis of bovine tuberculosis can be made following the macroscopic detection at necropsy of tuberculous lesions. Post-mortem examination is one of the

critical steps in the diagnosis of bovine tuberculosis prior to bacteriological confirmation of the disease by isolation of *M. bovis*. The sensitivity of gross post-mortem examination is affected by the method employed and type of the tissues and organs examined (Corner *et al.*, 1990; Corner 1994). For example, in Australia, regulations regarding post-mortem inspection were modified to enable detection of tuberculous lesions by incision of lymph nodes and tissues not previously incised or palpated (McMahon *et al.*, 1987). This change reduced the probability of missing an animal with tuberculous lesions by 47% (Corner *et al.*, 1990). The careful examination of at least 7 lymph nodes (*viz.* mediastinal, medial retropharyngeal, bronchial, parotid, caudal cervical, superficial inguinal and mesenteric lymph nodes) and lungs enabled Corner and others (1990) to detect 95% of the cattle with lesions. Although detailed post-mortem examination, which involves visual examination, palpation and slicing of 25 lymph nodes at 2mm thickness, and organs (*i.e.* lung, spleen and liver) at 20 to 50mm, as opposed to routine meat inspection, can improve detection rate, unfortunately, its applicability in ordinary abattoirs is limited and not cost effective (Corner *et al.*, 1990).

Histopathological examination of tissues suspected to have tuberculous lesions may increase the confidence of diagnosis and in the absence of bacteriological diagnosis this may be used as a standard for comparison with other detection methods (Corner *et al.*, 1990; Corner 1994).

1.1.6.3. Bacteriological diagnosis

Diagnosis of bovine tuberculosis by culture of material from live cattle has received little attention. This is attributed to the problem of obtaining relevant specimens for culture and also the length of time taken to grow *M. bovis*. Although, bovine tuberculosis in cattle is mainly pulmonary, cattle cough infrequently due to the nature of the pathology of the disease. In cattle, tuberculosis is mainly of the “closed type” (Lepper *et al.*, 1977; Pritchard 1988). Cultivation of *M. bovis* from cattle air ways has been demonstrated by Neill *et al.* (1988a). These workers achieved this by inoculation of guinea pigs with intranasal secretions from experimentally infected cattle, followed by culture of tuberculous material from sacrificed guinea pigs.

In the Netherlands, veterinarians were encouraged to obtain sputa from tuberculin reactor cattle for cultivation of mycobacteria (the sputum test). This activity was able to find 823 open lesions among 50, 000 cattle tested (Dr. L.M. O'Reilly personal communication 1996).

Excretion of *M. bovis* in faecal material from infected cattle has been documented by Stenhouse and Hoy (1930) and Maddock (1933). However, reports of isolation of *M. bovis* from cattle faeces are rare. Mark (1976) indicated that due to the presence of a heavy load of other contaminating bacteria, and presence of toxic material, isolation of mycobacteria from faecal material is undesirable.

Isolation of *M. bovis* from milk has been successful after initial *in-vivo* growth in guinea pigs (Collins and Grange 1983; Sinha 1994).

The commonest use of bacteriological diagnosis has been in the confirmation of post-mortem diagnosis where specimens from slaughtered cattle, such as lymph nodes and tuberculous organs, are cultured (Lepper *et al.*, 1977; Francis *et al.*, 1978; Corner *et al.*, 1990; Wood *et al.*, 1992). Corner (1994) has reviewed a number of precautions to be taken for successful isolation of *M. bovis*; these include, asepsis at the point of collection of lymph nodes, preservation of specimens during transportation, type of decontaminant used, type of medium for culture, and conditions and duration of incubation. *M. bovis* from primary cultures can be identified using set of biochemical tests described above.

1.1.6.4. Molecular diagnosis

Diagnosis of *M. bovis* by the use of recently developed molecular techniques is in its infancy period. As Collins and Grange (1982) and Hoffner *et al.* (1995) have suggested the use of molecular biology techniques would greatly improve the diagnosis of *M. bovis* not only in human subjects but also in cattle. The current detection and typing schemes described above are usually laborious and time consuming. Thus, the advent of the DNA based detection and typing techniques (*vide supra*; Chapter 4-6) have offered attractive alternatives to the conventional ones. Some of the PCRs described above (Table 1.5a&b) are now starting to be applied to detect *M. bovis* not only in cultures but also from tissues. Attempts to

develop a *M. bovis* specific PCR have been a subject of concern by many veterinary mycobacteriologists. Using the already developed PCR techniques for identification of the *M. tuberculosis* complex, Sinclair *et al.* (1995) developed an IS986/*mtp40* multiplex PCR which could discriminate *M. tuberculosis* from *M. bovis* and other species of the tuberculosis complex group. The development of a putative species-specific PCR for *M. bovis* is discussed elsewhere in this document (Chapter 5&6) Application of a PCR technique to bovine samples is reported by Spanish workers (Liebarna *et al.*, 1995). These workers were able to use IS6110 PCR to diagnose the presence of *M. bovis* in 71.4% of 81 tissues acquired from cattle in herds known to be infected by bovine tuberculosis. Wards *et al.* (1995) in Australia used a IS1081 PCR to amplify *M. bovis* DNA from 53 of 58, and five of 52 tissues from which positive and negative cultures were obtained respectively.

1.1.6.5. Diagnosis by animal inoculation

Animal inoculation as a means of diagnosing *M. bovis* infection is now an obsolete technique. It is only used as a amplification step for culturing clinical specimens with very low bacterial loads (Neill *et al.*, 1994; Sinha 1994). Comparison of pathology produced in rabbits and guinea pig after inoculation with tuberculosis suspected cultures or clinical material was used in the past to determine the species of tubercle bacilli in question. According to Colle *et al.* (1964), *M. bovis* infection produces more severe tuberculous lesions in rabbits than *M. tuberculosis*, whilst in guinea pigs, the lesions produced by *M. bovis* are no worse than those caused by *M. tuberculosis*.

1.1.7. EPIDEMIOLOGY OF BOVINE TUBERCULOSIS

1.1.7.1. Occurrence of bovine tuberculosis in cattle world-wide

Bovine tuberculosis is world wide problem, affecting approximately one percent of cattle in the North America, and Europe and 10 to 20% or higher in enzootic regions of Latin America, Africa and Asia (Steele 1995). The reflection of this infection rate on the world cattle population, which stands at 1 billion and with an average

prevalence of reactors a staggering 5%, means that more than 50 million cattle are affected (Steele 1995).

1.1.7.1.1. America

The countries making up the American continent have reported a varying incidence rate of the disease on the basis of the tuberculin test and/or post-mortem findings at meat inspection. In South America, de Kantor (1995) has summarised the results of tuberculin tests carried out in different countries between 1985 and 1990. She reported that the percentage of herds found to have reactors ranged from as low as 0% in Surinam to as high as 48% in Argentina. On the basis of meat inspection records, the percentage of cattle with tuberculous lesions ranged from 4.13% in Argentina, while there were no cattle with tuberculous lesions encountered in Surinam and Ecuador (de Kantor 1995).

In the Central America, the data presented by de Kantor (1995) have revealed that El Salvador had a high herd reactor rate (18.9%), while Panama and most of the Caribbean countries were free of tuberculosis on the basis of the tuberculin test. Regarding meat inspection records, the incidence was much lower; 0.73% of cattle slaughtered in Guatemala had tuberculous lesions, which is considered to be the highest in the region, whereas countries such as Anguilla, Barbados, Belize, Guadaloupe, Panama and Haiti did not detect any tuberculous lesions in cattle slaughtered between 1988 and 1989.

Reports from North America indicate that the United States and Canada are on the verge of eradicating bovine tuberculosis in domestic cattle (de Kantor 1995), with only a few reports of sporadic outbreaks of the disease on certain dairy farms in El Paso, United States (Shoebaum and Meyer 1995). However, the disease is still common in wildlife such as Bison, Elk, *et cetera* (Thoen and Himes 1981; Tessaro *et al.*, 1993).

1.1.7.1.2. Europe

The incidence of bovine tuberculosis in European countries has shown a downward trend as result of appropriate control measures instituted by individual countries

before and after the Second World War. Austria, Denmark, Finland, Luxembourg, the Netherlands, Norway, Sweden and Switzerland achieved a disease free status by the end of 1950's (Thoen and Steel 1995). Other countries such as Germany, France, Italy, UK, Hungary, Czech Republic, Romania and Poland have reduced the incidence of bovine tuberculosis to below 1% by 1980 (Caffrey 1994; Thoen and Steele 1995). European countries with problem herds i.e. an incidence rate above 1% are Ireland (3% in 1992) and Spain (3.1- 5.54% between 1978 to 1983) (Thoen and Steele 1995).

1.1.7.1.3. Africa

Bovine tuberculosis is prevalent in 33 of 41 countries in Africa (OIE-Report 1994; Chillaud 1995). Figure 1 illustrates the status of the disease up to 1990, a situation which is unlikely to have changed as only a few countries are instituting control measures (OIE - Report 1993/1994 - Chillaud 1995).

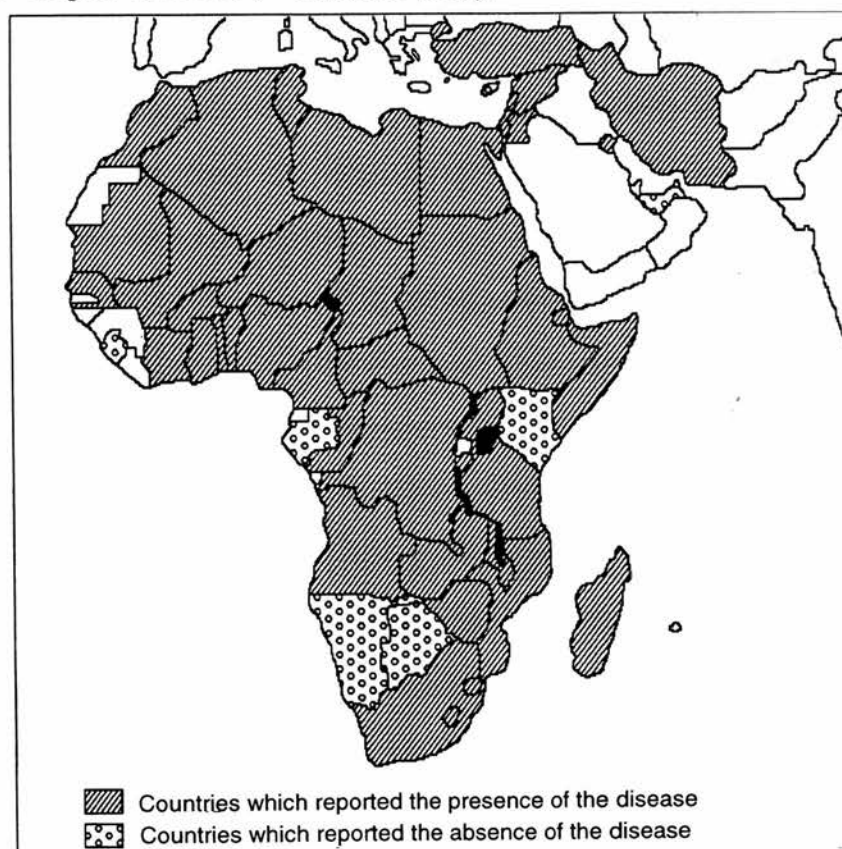


Figure 1.1. The status of tuberculosis in cattle in Africa and the Middle East 1986-1990 (Source Thoen and Steele 1995).

Precise data for individual countries are emanating from meat inspection records; this is due to the fact that there are no national wide programmes of tuberculin testing of cattle

1.1.7.1.4. Asia

The situation of bovine tuberculosis in the countries of the Middle East has shown a similar trend to African countries (Figure 1.1). Precise data are found only in countries reporting to the OIE (Thoen and Steele 1995).

The reports of the occurrence of bovine tuberculosis in India have indicated the presence of disease to vary from as high as 20.4% in Punjab and Bombay to as low as 2% in Madras and Bihar (Lall 1995). One report from Burma shows a prevalence rate of 8.8% (Langford 1995), whereas low rates of the disease incidence have been recorded by a number of countries of the Far East, China and Japan (0.01 - 1.6%) (Thoen and Steel 1995).

1.1.7.1.5. Oceania

Countries of Oceania, particularly Australia and New Zealand have recorded progress in eradication of bovine tuberculosis in cattle. Australia is practically free of tuberculosis, despite minor foci in the Northern Territories and Western Australia (Francis 1995). In New Zealand, the prevalence of bovine tuberculosis has dropped from 8.0% in 1960's when the control measures were started, to 0.2% by 1993; the remaining cases are due to the re-infection of herds declared free by *M. bovis* from wildlife reservoirs (particularly possums) (O'Hara 1995). Other countries in the Oceania region have either eradicated or have a very low level of the disease detected at meat inspection (Saville 1995; Wigglesworth 1995).

1.1.7.2. Occurrence of tuberculosis caused by *M. bovis* in animal species other than cattle

The occurrence of *M. bovis* in other animal species has been reported in a number of publications (Table 1.9). The epidemiological significance of *M. bovis* infection in other animal species arises from the fact that these animals may act as reservoirs for

tuberculosis which would complicate control and eradication programmes as has occurred in countries such as UK and Ireland, where badgers are implicated in herd breakdowns/outbreaks (Dolan and Hayden 1991; Noland and Wilesmith 1994), or in New Zealand, where the brush-tailed possum is regarded as a source of infection in a number of tuberculosis free herds (Collins *et al.*, 1983).

Table 1.9. Reported cases of tuberculosis due to *M. bovis* in animal species other than cattle

a. Domesticated animals

Animal species	Reporting country	Reference
Goat	UK	Griffith 1917
“	Uganda	Carmichael 1939
“	Tanganyika (Tanzania)	Milne 1955
“	India	Som and Bhattacharrya 1987
“	Australia	Cousins <i>et al.</i> 1993a
“	Spain	Bernabe <i>et al.</i> 1991
Sheep	New Zealand	Davidson <i>et al.</i> 1981
“	German	Cordes <i>et al.</i> 1981
“	India	Som and Bhattacharrya 1987
Pig	South Africa	Huchzermeyer <i>et al.</i> , 1992
“	USA	Thoen <i>et al.</i> 1986
Deer	Ireland	Dodd 1984
“	UK	Stuart <i>et al.</i> 1988
“	UK	Clifton-Hadley and Wilesmith 1991
“ (Elk)	Canada	Whiting and Tessaro 1994
“	USA	Morris <i>et al.</i> 1994;
“	Sweden	Bolske <i>et al.</i> 1995; Szewzky <i>et al.</i> 1995
Horse	UK	Stableforth 1924
“	USA	Lovel and white 1941
Camel	Egypt	Manson 1917
“	USA	Bleem <i>et al.</i> 1993
Mink	USA	Lovel and White 1941; Akay <i>et al.</i> 1984
Cat	UK	Gunn-Moore <i>et al.</i> 1996
“	USA	Snider and Cohen 1972
“	New Zealand	de Lisle <i>et al.</i> 1990
“	Tanzania	Dr. M. Mtambo personal comm. 1994
“	Sweden	Szewzky <i>et al.</i> 1995
“	UK	Blunden and Smith 1996
Dog	UK	Jenning 1949
“	USA	Snider and Cohen 1972
“	Nigeria	Ayanwale <i>et al.</i> 1983

b. Wild, zoo and captive animals

Animal species	Reporting country	Reference
Baboon	Kenya	Sapolsky and Else 1987
Badger	Europe	O'Reilly and Daborn 1995
Bison	USA	Thoen et al. 1988
"	Canada	Tessaro 1986; Tessaro <i>et al.</i> 1993
African buffalo	Uganda	Woodford 1982a
Cape buffalo	South Africa	Keet <i>et al.</i> 1994; Bengis <i>et al.</i> 1996
Feral buffalo	Australia	McCool and Newton-Tabrett 1979
Elk	Canada	Tessaro 1986
Feral goats	New Zealand	Sanson 1988
Kafue lechwe	Zambia	Gallagher <i>et al.</i> 1972
Great kudu	South Africa	Paine and Martihnaglia 1920
Feral pig	Oceania countries	Corner <i>et al.</i> 1981
Warthog	Uganda	Woodford 1982b
Wild rabbit	New Zealand	Gill <i>et al.</i> 1993
Hare	New Zealand	Cooke <i>et al.</i> 1993
"	Argentina	de Kantor <i>et al.</i> 1984
Possum	New Zealand	Tweddle and Livingstone 1994
Seals	Australia	Cousins <i>et al.</i> 1993b
Monkey	Canada	Hariharan 1988
Foxes	USA	Thoen 1988
Cheetah	South Africa	Keet <i>et al.</i> 1996
Lion	South Africa	Keet <i>et al.</i> 1996
Rhinoceros	USA	Dalovisio <i>et al.</i> 1992
Tiger	USA	Lumeij <i>et al.</i> 1987

1.1.7.3. Transmission of *M. bovis* to cattle and other animals

The success of transmission of any disease is a result of successful interaction between host, agent and the environment (Thrusfield 1986). In the case of bovine tuberculosis, host factors associated with risk of disease include the breed of cattle. For example, Zebu cattle (*Bos indicus*) are regarded to be naturally resistant (unless stressed) to bovine tuberculosis, whereas *Bos taurus* (European breeds) are regarded to be fairly susceptible to *M. bovis* infection (Radostits *et al.*, 1994). Regarding agent factors, *M. bovis* is moderately resistant to heat, desiccation and many disinfectants, but can be readily destroyed by direct sunlight, unless in a moist environment (Radostits *et al.*, 1994). Environmental factors, which may augment the occurrence of the disease include housing and management practices, such as over crowding of

cattle in-doors, congregation of cattle at feedlots or at drinking ponds during the dry season (Radostits *et al.*, 1994).

1.1.7.4. Source of infection

The spectrum of natural hosts of *M. bovis* has been indicated above (Table 1.9) and in a recent review on the epidemiology of *M. bovis* infection in animals and man (O'Reilly and Daborn 1995). The hosts include humans, non-human primates, cervidae, wild and domestic ruminants, equines, porcines, camelidae, badgers, possums, ferrets, seals, and lagomorphs.

It has also been shown that *M. bovis* can survive in a number of inanimate objects and on pasture following contamination. Maddock (1933) showed that tubercle bacilli present in the discharged pus or other morbid material can survive in the environment for several weeks, and also indicated that bacilli excreted in faeces may remain viable for over five months. Furthermore, it was reported that water, artificially contaminated with *M. bovis*, may be infective for up to ten weeks (Maddock 1933). Wray (1975) observed a variation in duration of survival of *M. bovis* in faeces with the season of the year as follows: in winter, 5 months; autumn, 4 months; and summer, 2 months. In experimental studies on the effect of sunlight on survival of *M. bovis* in dry and moist soil conditions, Duffield and co-workers (1985) revealed that *M. bovis* could survive in these milieu under the shade and in darkness, whereas no *M. bovis* survived when these milieu were exposed to sun light. Vera (1984) found that *M. bovis* can survive in the sediment at the bottom of a flask of water for 245 to 295 days under winter temperatures, while its survival in water under summer conditions is 203 to 237 days. These observations indicate the potential of water as a source of *M. bovis* for cattle.

Infected cattle have been demonstrated to be the main source of infection to susceptible cattle (Steger 1970; Neill *et al.*, 1989/1992). It requires at least 87 days for cattle naturally infected with *M. bovis* to start to excrete the organism (Neill *et al.*, 1991). However, unless stressed, cattle may remain free of tuberculosis for a duration of four to nine months of contact with infected cattle (O'Reilly and Costello 1988).

Transmission of the tuberculosis from man to cattle has been documented (Cutbill and Lynn 1944; Leslie 1968; Huitema 1969; Corner and Lepper 1983). Respiratory infection as a result of close contact between infected cattle-owners and their cattle has been speculated, and in one exceptional report, the oral route was implicated. An infected owner had a habit of urinating in the hay barn, and this hay was later fed to his cattle (Huitema 1969).

1.1.8. ZOONOTIC IMPLICATIONS OF *M. BOVIS*

The investigations carried out by the British Royal Commission in 1911, the German Commission (Kaiserliche Gesundheitsamt) in 1908, and the New York Department of Health Commission in 1911, all concluded that man must be added to the list of animals notably susceptible to bovine tubercle bacilli and that there was reciprocal transmission of the disease between cattle and man (Grange 1995). Since that time numerous reports have emerged indicating infection of *M. bovis* in man (see below).

In 1908, Koch modified his views on the infectious nature of the bovine tubercle bacillus. He indicated that pulmonary tuberculosis was more important compared to other forms of the disease, and furthermore he indicated that no cases of pulmonary disease due to bovine tubercle bacillus had ever been reported (Grange and Collins 1983). However, Griffith (1937) started to notice cases of pulmonary tuberculosis due to bovine tubercle in Great Britain. The proportion of these cases to total tuberculosis cases ranged from 0.5 -0.6% in Southern England to as high as 8.5% in Scotland (Griffith 1937). Similar figures were encountered in a number of European countries, with the rural areas being most affected (Ruys 1939; Sigurdsson 1945).

The major anatomical sites of involvement of tuberculosis caused by bovine tubercle bacilli in man as reported by Griffith (1937) included the lymph nodes of the neck region, bones and joints, abdomen and skin, genitourinary disease by contrast was rare. The preponderance of infection to specific anatomical sites has changed with time. For example, in Denmark, 30.7% of total *M. bovis* cases were pulmonary between 1931 to 1935 and rose to 58% in between 1958 and 1963 (Torning 1965). In UK, in a survey of *M. bovis* in the South East England (1977-1990) it was shown

that up to 40% of the isolates originated from pulmonary cases and among the non-pulmonary cases the genitourinary was the most common (38%) as compared to 27% isolations from lymph nodes (Grange and Yates 1994).

The review of Kleeberg (1984) has indicated that extra-pulmonary tuberculosis is a consequence of invasion of blood stream by tubercle bacilli from exudative lesions in the primary complex, and further explained that involvement of non-pulmonary organs is sequel to infection via the oral route rather than the affinity of the organisms for certain abdominal organs. Infection of tissues remote from the primary complex have been recorded. These include skin tuberculosis or 'tuberculous verrucosa cutis' (Hruza and Snow 1990; Healy and Rodgers 1992), tuberculous mastoiditis (Smith and Anders 1994) and tuberculous meningitis (Norton *et al.*, 1995).

Current reports on the world trend of tuberculosis indicate that this disease is a more common cause of death in adults than AIDS, malaria, diarrhoea *etc.*, with approximately 3 million deaths per year (Styblo 1981; Rodrigues and Smith 1990). The contribution of *M. bovis* to tuberculosis morbidity and mortality has not been clearly stipulated. This shortcoming arises from a number of factors but the main one is the lack of enthusiasm for typing strains of the tuberculosis complex by many mycobacteriology laboratories and physicians seldom will request such information after submission of specimens. The bulk of information, therefore, is a result of particular studies rather than from the surveillance data gathered by mycobacteria laboratories. For example, in Great Britain, between 1977-1981, the Public Health Laboratory Service (PHLS) identified 566 isolates of *M. bovis* but only 125 (22.5%) were presented to Communicable Disease Surveillance Unit by physicians or microbiologists (Grange and Collin 1987). A similar situation has been reported in the USA. (Habib and Warring 1966) as well as in Canada (Wigle *et al.*, 1972). A list of some of recent publications of reported cases of *M. bovis* infection in man is presented in Table 1.10.

1.1.8.1. Transmission of *M. bovis* from cattle to man

1.1.8.1.1. Oral route

The mode of transmission of *M. bovis* from cattle to man was the subject of investigations by the commissions set up in Britain, Germany and USA, all of which realised the potential of *M. bovis* as a pathogen of man. There are a number of ways through which man can acquire the infection. Firstly, consumption of milk; as stated by Kleeberg (1984), in the old days, 'child TB was equal to bovine TB', and drinking unpasteurised milk was implicated in many of those cases. Evidence of excretion of *M. bovis* in the milk of infected cattle was reported by Sigurdsson (1945). This report listed a number of isolations of *M. bovis* from milk samples in a number of European countries prior to and during bovine tuberculosis eradication campaigns (Sigurdsson 1945).

Although legislation has been passed for pasteurisation of milk in most countries in the developed world, in the majority of countries of Africa, Latin America, and Asia, such measures are lacking. The custom of drinking unpasteurised milk still persists, and even in those countries where milk is boiled, milk intended for making yoghurt and cream is normally not boiled.

Transmission of *M. bovis* to man through consumption of contaminated meat has been a subject of controversy. The Mosaic laws, as stipulated in Leviticus XXII:27, contain early rules which prohibit the use of tuberculous animals for food; similar laws were enacted in Germany and France in the early part of this century (Moore 1913). There is no clearly understood mode through which tuberculosis in tissues and organs consumed by man can cause infection in man. The studies of M'Fadyean (1892) and Francis (1958) failed to initiate the disease in many of the rabbits and guinea pigs fed raw minced beef from infected cattle, and Francis (1973) noted that although in the course of mycobacterial infection, bacillus could spread haematogenously to other parts of the body including muscles, he stated that the chance of transmission of *M. bovis* through the consumption of meat was very slim.

Table 1.10. A list of publications of the isolation of *M. bovis* from human cases of tuberculosis

Country	Isolation rate <i>M. bovis</i> /MTBC* (%)	Distribution of <i>M. bovis</i> cases		Reference
		PULM. ^a	EXTRA ^b	
Canada	31/6322 (0.5)	13	18	Wigle <i>et al.</i> 1972
“	57/14,731 (0.004)	57	-	Liss <i>et al.</i> 1994
U.K	77/2751 (2.8)	20	57	Wilkins <i>et al.</i> , 1986a&b
“	201/16,862 (1.2)	81	120	Yates and Grange 1988
Scotland	33/3976 (0.83)	10	23	Barrie and Bruce 1966
Nigeria	4/102 (4.0)	4	-	Idigbe <i>et al.</i> 1986
E. Germany	169/4507 (3.7)	169	-	Kappler <i>et al.</i> 1986
Argentina	49/13544 (0.36)	NS	NS	Barrera <i>et al.</i> 1987
Russia	157/689 (22.8)	-	157	Kovalyov 1989
Australia	87	67	20	Georghiou <i>et al.</i> 1989
New Zealand	22/305 (7.2)	7	15	Brett 1991
Ireland	17/263 (6.4)	12	5	Cormican <i>et al.</i> 1992
“	30/656 (4.8)	16	14	Cotter <i>et al.</i> 1996
USA	73/2430 (3.0)	28	45	Dankner <i>et al.</i> 1993
Sweden	96/4822	NS	NS	Anon 1994
Malawi	1/30 (3.3)	1	-	Parry <i>et al.</i> 1995
Ethiopia	1/44 (2.2)	1	-	Yehualaeshet 1995

- specimen not collected NS - not specified * MTBC = the *M. tuberculosis* complex ^a Pulmonary cases ^b Extra-pulmonary cases

Pritchard (1988), complemented this information by indicating that meat is usually cooked before consumption, hence killing many pathogenic micro-organisms, and therefore limiting the transmission of *M. bovis* to man via eating meat.

1.1.8.1.2. Respiratory route

Cutbill and Lynn (1944) provided a record of suggested airborne transmission of *M. bovis* from cattle to man. In their investigation of 48 cases of *M. bovis* infection in

man, they found out that 21% of the cases may have arisen from the aerogenous route as result of close contact between people and cattle. Jensen (1953), noted that the airborne transmission of *M. bovis* from cattle to attendants is very likely, as they were able to recover acid-fast bacilli from the walls and windows of the shed housing infected cattle. Aerogenous transmission of *M. bovis* has been recorded in a number of occupationally exposed of people working with various kinds of animal species not only cattle. For example, i) the outbreaks of pulmonary *M. bovis* infection among abattoir workers in Australia (Robinson *et al.*, 1988; Georghiou *et al.*, 1989), ii) the isolation *M. bovis* and conversion to skin-test positive among veterinary attendants in contact with *M. bovis* infected slaughter elk in Canada (Fanning and Edwards 1991), iii) in USA zoo workers converted to skin-test positive after being in contact with a white rhinoceros infected with *M. bovis* (Dalovisio *et al.*, 1992; Stetter *et al.*, 1995), and iv) in the Netherlands, an alpaca was implicated in *M. tuberculosis* infection in an animal caretaker and laboratory technician who attended the autopsy of the alpaca from which *M. bovis* was isolated (Veen 1991).

1.1.8.1.3. Miscellaneous route

Butcher's wart (Skin tuberculosis), is a common condition amongst butchers and slaughter house workers who handle tuberculous carcasses. Infection is normally acquired via cuts and abrasions on the skin (Grange *et al.*, 1988).

1.1.8.2. Man to man transmission of *M. bovis*

Dissemination of bovine tubercle bacilli by man would be possible if an infected person with pulmonary *M. bovis* infection is in contact with a susceptible individual as occur with human type bacillus. The pathology and epidemiological data gathered from some of the cases examined by Griffith and Munro in 1935 suggested familial transmission of bovine tubercle bacilli from an infected adult to an adult and a child in two families (Griffith 1937). Cutbill and Lynn (1944) also found that 3% of the 48 cases they examined might have been a familial spread of infection. Apart from these old reports, many authors have suggested that it is difficult to prove the existence of man to man transmission of *M. bovis* infection in the presence of disease in animals

within the same geographical area (Grange and Collins 1987; Hardie and Watson 1992; van Soolingen *et al.*, 1994). However, the use of molecular biology typing techniques enabled van Soolingen *et al.* (1994) to establish man to man transmission of *M. bovis* in Amsterdam, the Netherlands. In their study on the use of various genetic markers in differentiating strains of *M. bovis*, they encountered 5 strains which showed the same banding pattern, 3 were isolates from members of the same family, one from a person living within the same dwelling, and one from a distant person without contact to the former cases. Similar techniques enabled the trace back of a multidrug resistant *M. bovis* infection amongst patients in an AIDS ward in France (Bouvet *et al.*, 1993).

1.1.8.3. Man to cattle transmission of *M. bovis*

Concern is now raised of the possibility of the so-called reverse zoonosis, where cattle might be infected with human-derived *M. bovis* in the wake of the HIV/AIDS era (Cosivi *et al.*, 1993; Daborn and Grange 1993). The early indications that human-derived tubercle bacilli might infect cattle arise from a publication by Griffith (1917). The author indicated that cattle vaccinated with human type bacilli as a preventive measure secreted virulent bacilli in milk and had non-progressive lesions in their tissues. Further evidence of man to cattle transmission of tubercle bacilli came from the investigation of Lesslie in 1950's, where a total of 17 cases of cattle reacting to tuberculin in attested herds had arisen from herds where there was an infected owner or attendant (Lesslie 1968). In another study in Germany, Weber *et al.* (1988) found that in 16 of the 49 cattle herds, where re-infections had occurred, there were human cases of *M. bovis* infection. Among the human cases, with exception of two, all were over 60 years of age, and that 70% were pulmonary and 30% renal tuberculosis. Huitema (1969) indicated that cattle were infected with *M. bovis* as result of consumption of hay which was urinated on by the cattle keeper and owners. Urinating on hay bales by some farmers was regarded as means of increasing nourishment to cattle feed (Huitema 1969).

1.1.8.4. Association of *M. bovis* infection and HIV/AIDS epidemics in man

According to Sunderman *et al.*, (1986), tuberculosis was not regarded as a major disease associated with HIV/AIDS in either the USA or Europe until it started to appear in ethnic minorities and intravenous drug users in USA. In developing countries, however, tuberculosis was, and is still, regarded as the most common opportunistic infection in patients with HIV/AIDS. In Zimbabwe (McLeod *et al.*, 1988) and in Ethiopia (Lester *et al.*, 1988) it has been shown that up to one third of AIDS cases are co-infected with tuberculosis. A similar situation has been encountered in other countries South of the Sahara (Porter 1991). It is not clear if tuberculosis in HIV/AIDS patients is as a result of primary, reactivated or secondary exogenous infection (Harries 1990) but, whatever the source, it has been shown that, the progression of tuberculosis is accelerated by up to 8% by HIV/AIDS (Selwyn *et al.*, 1989). A world wide estimate of tuberculosis cases with dual infection of HIV stands at 3.9%, but in Sub-Saharan Africa the figure stands at 17% (Kochi 1991). The extent to which *M. bovis* infections in man are associated with HIV/AIDS has not been fully elucidated all over the world (Cosivi *et al.*, 1993; Daborn and Grange 1993; Drobniewski *et al.*, 1995). There are only a few reports of sporadic incidence of *M. bovis* infection in HIV/AIDS cases. Dankner *et al.* (1993) reported isolation of *M. bovis* from 4.2% and 45.8% of patients with HIV/AIDS manifesting pulmonary and non-pulmonary forms of tuberculosis respectively. Bouvet *et al.* (1993), reported on a nosocomial outbreak of multi drug resistant *M. bovis* among HIV infected patients, where a single index case was responsible for transmitting infection to five other patients and one health care worker. Apart from reports on outbreaks, there is a number of reports describing single cases of this dual infection (Smith *et al.*, 1992; Marks *et al.*, 1993; Albercht *et al.*, 1995). Despite the paucity of information regarding dual infections of *M. bovis* and HIV/AIDS in the developing world, it is envisaged that the occurrence of such cases may constrain the already over-stretched resources used in the control of human tuberculosis as well as in cattle (Harries 1990; Bouvet *et al.*, 1993; Daborn and Grange 1993).

1.1.9. CONTROL OF *M. BOVIS* INFECTION IN CATTLE AND MAN

1.1.9.1. Control of bovine tuberculosis in cattle

1.1.9.1.1. Preventive measures

Control of bovine tuberculosis in cattle dates back to Koch's discovery of the tubercle bacillus. In 1883, McFadyean initiated measures which were directed towards protection of the human population from acquiring tuberculosis from cattle. The method he employed was based on detection of clinical disease, bacteriological examination of milk and, occasionally, tuberculin testing followed by voluntary slaughter of affected cattle (Pritchard 1988). A similar approach was applied in Germany, where Ostertag believed that the major source of infection was clinically diseased cattle. Diseased cattle were slaughtered and, at the same time, calves were separated from dams, fed with milk from sound cows or pasteurised milk from infected cattle. These measures were applied by Prussian Government in 1933 and were found to reduced the prevalence of the disease from 20% to 15% in three years (Francis 1947).

Toward the end of 19th century, Professor Bernhard Bang began significant work directed at eradicating bovine tuberculosis from cattle in his native country, Denmark. Bang's efforts paved the way to the introduction of legislation which demanded pasteurisation of all skimmed milk for stock feeding, notification and killing of every cow with tuberculosis of the udder. In addition, encouragement for farmers in combating tuberculosis among cattle comprised free tuberculin tests for farmers willing to separate reactor cattle from tuberculin negative animals - 'The Separation Method' (Sigurdsson 1945). Although the idea was sound, farmer co-operation was minimal and hence the scheme did not achieve the expected goal (Pritchard 1988). However, this idea was taken into consideration by a number of other countries which introduced a test-and-slaughter policy, which in its infancy was voluntary but later became compulsory (Francis 1947/1958; and Myers and Steele 1969). The outcome of the test-and-slaughter policy is the eradication of bovine tuberculosis in most West European countries, with exception of UK, Ireland, Spain, France and Italy, where sporadic cases are still prevalent (Caffrey 1994). Similar

disease free status has been or is nearly achieved by other developed countries outside Europe, such as USA, Canada, Australia and New Zealand (Thoen and Steele 1995). Efforts to develop a vaccine for combating tuberculosis in cattle also started at the end of the 19th century, after Koch's submission that human type tubercle bacilli could be used to immunise cattle against tuberculosis. The vaccine was used for a number of years until Griffith (1913) showed that vaccinated cattle were actually secreting human type tubercle bacilli in cattle milk. Subsequently, the use of this vaccine was abandoned. The development of a safe vaccine was achieved by French scientists, Calmette and Guérin. They passaged a strain of *M. bovis* up to 300 times on bile salt containing medium from which emerged an avirulent *M. bovis* strain termed *M. bovis* BCG (Calmette and Guérin 1909). This strain was then distributed to a number of laboratories world wide for making human vaccine. Other vaccines that were developed were the Graub vaccine from *M. bovis* (strain Vallee) and one from *M. microti* (the volle bacillus) (Pritchard 1988).

Several experiments on the use of BCG vaccine for cattle have been carried out by a number of workers with varying success. The early use of BCG vaccination of cattle in France was successful until 1930, when a mix-up of cultures occurred in the laboratory resulting in release of a virulent strain of *M. tuberculosis*, and subsequent abandonment of use of the vaccine - 'The Lubeck disaster' (Pritchard 1988). In Lubeck, Germany, 72 infants died of fulminating tuberculosis within a few months of inoculation, supposedly with the wrong strain (Anon 1980). In a review by Francis (1947/1958) and Schliesser (1972) on the use of BCG vaccine in cattle, they found that the vaccine offered a short-lived immunity, which would therefore not contribute to the control of bovine tuberculosis. Further evidence that BCG vaccine was not protective in cattle was presented by Larson and Evans (1929), Doyle and Stuart (1958), Berggren (1978) and Moodie (1977;1981). In view of the fact that vaccination was not successful in eradication of the disease, the Joint WHO/FAO Expert Committee on Zoonoses (1959) passed a resolution that vaccination had no place in the eradication of tuberculosis in cattle. There are, however, reports of the successful use of BCG vaccine in control of tuberculosis in cattle in various countries

(Brinet 1929; Gorov 1954; Rolls and Wiethe 1956; Ellwood and Waddington 1972; Waddington and Ellwood 1972).

Control of bovine tuberculosis by vaccination became obsolete in most European countries after the adoption of test-and-slaughter policies as it was found that *M. bovis* BCG would sensitise cattle in a similar manner to virulent *M. bovis* (WHO/FAO 1959). In spite of that, vaccination seems to be the only affordable option that developing countries may adopt as a control strategy. This is emanating from the fact that most of these countries are not able to carry out a test-and-slaughter policy due to limited resources for compensation to farmers. Furthermore, fresh evidence indicates that cattle, particularly calves, may be successfully protected by BCG vaccine depending on the source of vaccine (Buddle *et al.*, 1995a&b). It has been noted (O'Reilly and Daborn 1995) that the failure of trials in Malawi (Berggren 1977;1981) might have been as a result of use of British (Glaxo) BCG vaccine, which has been shown to lack a major immunoreactive protein (MPB70) secreted by *M. bovis* and most strains of *M. bovis* BCG.

M. vaccae has been used experimentally as a vaccine in man with varying success (Stanford and Stanford 1994). Trials of vaccination using *M. vaccae* and later on challenge with virulent *M. bovis* in experimental calves by Buddle and co-workers (1995b) have found this vaccine not to be protective.

1.1.9.1.2. Chemoprophylaxis

The discovery of antituberculosis drugs in the 1940's provided the medical profession with additional ammunition to combat human tuberculosis (Mitchison 1985). In order to effectively cure a patient with tuberculosis, there are two regimes, the traditional long-course treatment which covers a total of 18 to 24 months or a recently introduced short-course chemotherapy, which requires a total of six months of intensive treatment (Fox 1980). The major problem with long term treatment has been compliance of patients to complete the treatment regime and improper use of the drugs, especially in the developing world (Murray 1990). However, in the developed countries, there have been major successes as manifested by an increased rate of decline of mortality due to tuberculosis by 10% per year (Bates 1993).

The application of chemoprophylaxis in control of bovine tuberculosis in livestock and zoo animals has received little attention due to the costs and logistical problems of its administration (Pritchard 1988). In spite of that, there have been isolated situations where chemoprophylaxis has been the mode of control of disease in precious animals. For example, in South Africa, where isoniazid was used to treat dairy cattle (Kleeberg 1967) and in Saudi Arabia, an outbreak of *M. bovis* infection among captive Arabian oryx was successfully contained by treating infected oryx with a combination of ethambutol hydrochloride, rifampicin and isoniazid (Greth *et al.*, 1994). Isoniazid therapy has also been prescribed for a number of primates found to be suffering from tuberculosis in a number of zoos in the USA. (Thoen and Himes 1982).

1.1.9.2. Control of *M. bovis* infection in man

The WHO has estimated that tuberculosis would be eliminated in the developed countries by the year 2000. This supposition arose from the success of control measures which comprised immunisation using BCG and effective treatment of new cases. The contribution of *M. bovis* infection to human tuberculosis has been discussed in this chapter. It appears that control of human tuberculosis would not be complete without instituting measures to control other sources of infection, such as cattle derived *M. bovis*. Measures such as pasteurisation of milk were specifically introduced to protect the human population from milk-borne disease, particularly tuberculosis and brucellosis (Bloom and Murray 1992; Collins 1994). BCG vaccination is protective not only to *M. tuberculosis* but also *M. bovis*; this vaccine is currently being used world wide except in North America.

Currently used drug treatment regimes are effective in combating *M. bovis* infection in man. The only exception is that *M. bovis* is naturally resistant to pyrazinamide, the drug used in combination with others in the short-course chemotherapy (Scorpio and Zhang 1996).

One of the major purposes of instituting control measures for tuberculosis in cattle, has been to reduce tuberculosis in man. Considerable achievement has been made in the developed countries, where at the present moment, human tuberculosis due to *M.*

bovis mainly results from reactivation of old infections and thus mainly confined to old people rather than exogenous infection of the young generation (Hardie and Watson; Grange 1994). The situation in developing countries is difficult to assess as there is a general paucity of data regarding *M. bovis* infection in man.

1.2. BOVINE TUBERCULOSIS IN TANZANIA

1.2.1. BACKGROUND INFORMATION

Bovine tuberculosis in Tanzania was first seen after the First World War amongst imported cattle in Dar-es-Salaam (Hornby 1949). These cattle were from South Africa. Prompted by the high incidence of tuberculosis in the Wachagga tribe Cornell (1934) conducted a survey of their indigenous cattle. Of the 441 animals tested, 8 reacted positively to mammalian PPD; 4 of these reactors were slaughtered but at necropsy all were negative. Markham (1952) reported a number of observations that confirmed the presence of the bovine tuberculosis in Tanzania and a recent report (Maiseli *et al.*, 1989) has confirmed the presence of bovine tuberculosis amongst cattle in the Southern Highlands of Tanzania. Meat inspection records from one of the districts at the centre of the Usangu Plains also indicate the presence of tuberculous lesions in 20% of the cattle slaughtered (Table 1.11; Mr. Nzobanaliba, Mbarali Sub-District Livestock Officer - personal Communication 1992).

Table 1. 11. Organs condemned due to bovine tuberculosis in Mbarali Sub-District for the year 1991*.

Types of organs	Number of organs condemned	Percentage of total slaughtered cattle**/year
Lungs	602	18.9%
Small Intestines	100	3.2%
Heads	33	1.0%
Livers	105	3.3%
Whole Carcass	14	0.4%

* Source Mbarali Sub-District Meat Inspection Report for 1991 ** n =3170

1.2.2. EPIDEMIOLOGY OF BOVINE TB IN TANZANIA

The epidemiology of bovine tuberculosis in Tanzania cattle is still not very well understood. One of the major reasons for this shortcoming is the lack of proper disease surveillance systems in the country (Mbassa and Kazwala 1993). Although testing of animals for tuberculosis was carried out prior to independence in particular areas within the country, the work done thereafter was mainly confined to the dairy farms belonging to the government and its institutions.

In Tanzania, disease surveillance is carried out by Veterinary Investigation Centres (VICs), which are situated in 7 geographical zones (Figure 2; Table 1.12). The limited work done by these VICs in tuberculin testing cattle, revealed the presence of disease in the Southeast and Northwest regions in addition to those reported by Markham (1952) (Table 1.12). The policy of testing government farms was continued until the mid 1980's, when the programme collapsed. This was attributed to, among many factors, a lack of resources for staff emoluments, lack of reliable transport to distant places and lack of antigens. Though a commendable job was carried out by the VIC'S, there were two major short falls, (i) the animals tested were grade or pure exotic breeds belonging to state farms which comprise only 1-2% of the national herd and (ii) information of confirmation of diagnosis by bacteriology and pathology was very scarce. The latter could be due to the lack facilities to culture *Mycobacterium* spp. Therefore, verification of the diagnosis using the tuberculin test could not be achieved in Tanzania.

The diagnosis by post-mortem examination of slaughtered cattle has, and will continue to, provide a core of information regarding the status of bovine tuberculosis in Tanzania. This information, though valuable and to certain extent reliable, has a number of shortcomings as follows:- (i) Often the ability of meat inspectors to diagnose tuberculous lesions especially at slaughter slabs in rural areas, is questionable. Problems of differential diagnosis, involving such diseases as actinobacillosis and the presence of abscesses in tissue and organs, may complicate diagnosis.

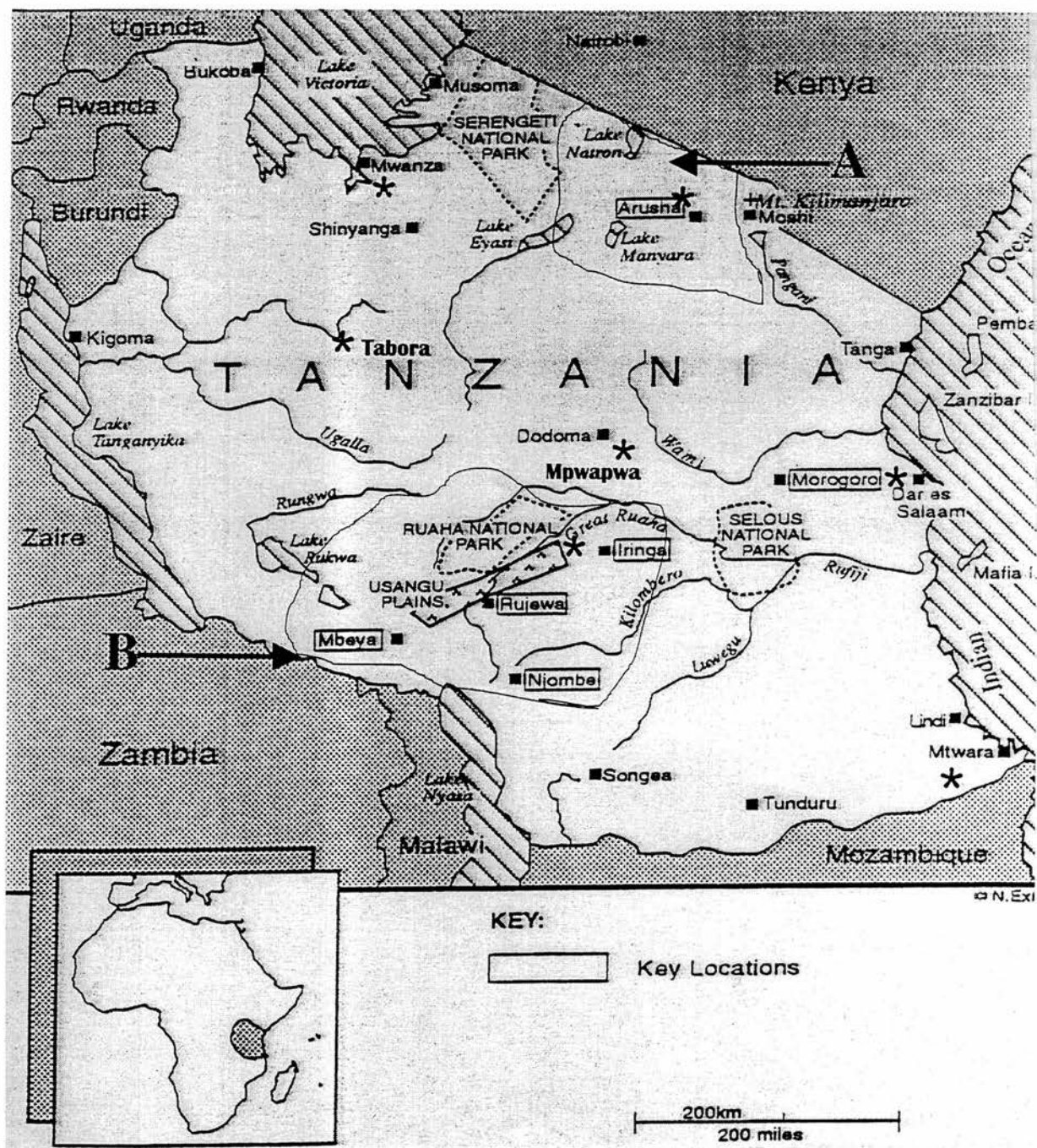


Figure 1.2. Map of Tanzania indicating project areas A (Arusha- Northern Zone) and B (the Southern Highlands Zone), and locations of VIC (*).

(ii) Problems associated with design and functioning of slaughter houses and slabs. Often there is congestion of butcher staff handling carcasses, lighting problems and livestock owners witnessing the inspection and influencing the decision. (iii) Inaccuracy of the reporting system - there is no systematic way of recording findings at most of the slabs (iv) lack of laboratory facilities to confirm post-mortem findings by even ZN staining of smears for acid fast bacilli.

Table 1.12. Results of Zonal tuberculin testing in Tanzania (1975 - 1990)*

Zone (name of VIC)	Total cattle tested	Positive reactors (%)	Inconclusive (%)	Negative (%)
Southern Highlands (Iringa)	6061	57 (0.57)	111 (1.83)	5893 (97.23)
Northern (Arusha)	3132	24 (0.77)	0 (0.0)	3099 (99.23)
Southern (Mtwara)	566	67 (11.84)	54 (9.54)	445 (78.62)
Eastern (Dar es Salaam)	1710	19 (1.11)	0 (0.0)	1691 (98.99)
Central (Mpwapwa)	522	1 (0.19)	0 (0.0)	521 (99.89)
Western (Tabora)	69	2 (2.90)	0 (0.0)	67 (97.10)
Lake** (Mwanza)	8190	18 (0.21)	326 (3.98)	7583 (92.5)

* Source - Salum (1993) cited by Chillaud (1995)

** Source - Prof. S.F. Jiwa - personal communication (1995)

1.2.3. ZOONOTIC IMPLICATION OF BOVINE TB IN TANZANIA.

Tuberculosis in man due to *M. bovis* generally occurs as the extra-pulmonary form, in particular cervical lymphadenitis (Jensen, 1955; Sjorgern and Sutherland, 1974; Kovalyov, 1989; Brett and Humble, 1991). In Tanzania, the proportion of extra pulmonary TB amongst all forms of tuberculosis stands at nearly 16 % (NTLP 1992 Report). The major part of these cases have been recorded in the Arusha region, in the north of Tanzania, where regional data indicate up to 30% of total tuberculosis cases are the extra-pulmonary form (Kazwala *et al.*, 1993). Early reports suggestive of *M. bovis* infection in the human population dates back to the 1930's, where a report by Cornell (1934) indicated the presence of tuberculosis amongst Wachagga

who were living underground with their cattle. Wilson (1954) also noted that for the Barbaigs, "what is injurious to health is the habit of keeping all young stock in the women's hut, where the young children also sleep. Bovine diseases such as ringworm, tuberculosis and anthrax can be carried in this way". At present there is a high incidence of extra-pulmonary tuberculosis in the Arusha region in the North of Tanzania (NTLP 1992 Report), where the main ethnic groups comprise Maasai, Iraqis and Barbaigs who form the majority of patients diagnosed to have extra-pulmonary forms of tuberculosis (Kazwala *et al.*, 1993; Dr. Swai - Arusha Regional TB/Leprosy Co-ordinator personal communication 1992). The predisposing factors for this condition in the aforementioned ethnic groups are the close contact between man and cattle, some of the groups keep their stocks indoors, and also the custom of drinking raw milk and blood.

Grange (1992) has suggested that the role of *M. bovis* in TB patients should be determined as a matter of urgency, particularly in Africa where bovine TB is still endemic. However, the second edition of the NTLP manual (1987), stipulated that "in Tanzania tuberculosis is due in nearly every case to human strains of tubercle bacilli; infections caused by *M. bovis* (through milk) are rare". The problem in proving the presence of *M. bovis* in man has many facets. One, for example, is the lack of interest amongst medical physicians/microbiologists in requesting typing of strains isolated from primary culture on IUT/L-J pyruvate media. Their primary interest is recovery of tubercle bacilli rather than knowing the species concerned. For example, when Fison (1986) was discussing the implications of bovine tuberculosis with medical personnel in Mtwara, he noted that "Medical workers barely consider, and indeed are extremely ignorant about, bovine tuberculosis in the context of public health". The other problem is lack of interest in taking specimens from extra-pulmonary cases for culture. As a consequence, the diagnosis is biased to reveal the presence of *M. tuberculosis* infection rather than other members of the tuberculosis complex. In view of the aforementioned shortcomings, some authors have used the figures of extra pulmonary tuberculosis as an indication of the extent of *M. bovis* infection (Kleeberg 1984).

In Tanzania, the increasing cases of extra-pulmonary tuberculosis paralleled the increasing total cases of tuberculosis reported each year between 1983 - 1991 (Figure 1.3; NTLP - report 1992). Catley (1992) and Kazwala *et al.* (1993) demonstrated a positive correlation ($r=0.67$) between the proportion of extra pulmonary tuberculosis and cattle to human population ratio (Figure 1.4).

According to Grange and Daborn (1995) the increase in bovine tuberculosis could be associated to a certain extent with the following factors: declining living standards in villages, increased urban and peri-urban cattle keeping, increased size and density of the urban population, improved detection and most notably, the emergence of the HIV/AIDS virus among people leaving in urban and peri-urban areas.

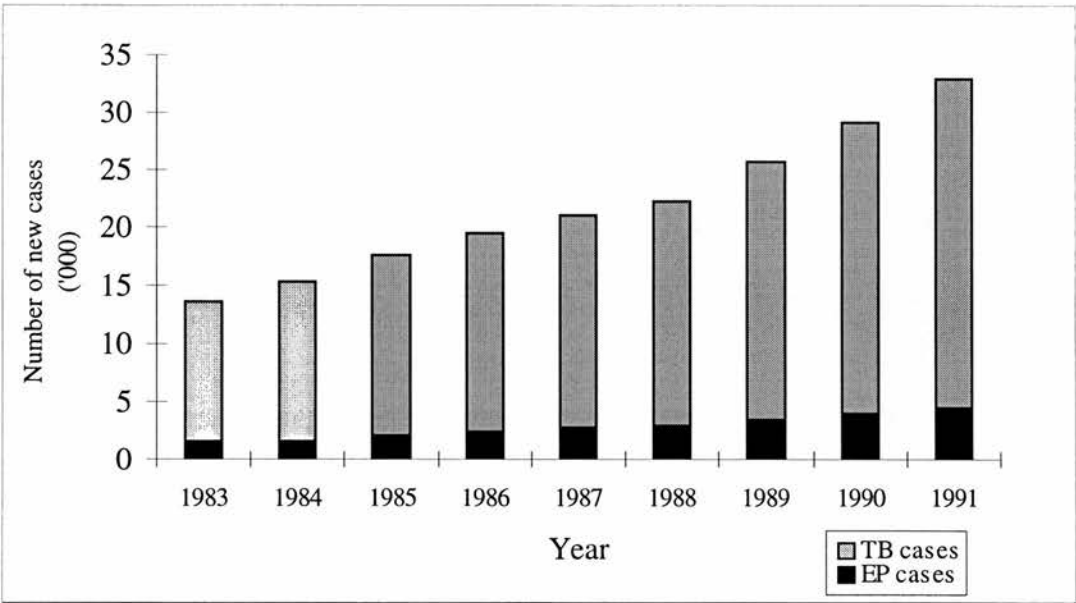


Figure 1.3. The trend of tuberculosis in man in Tanzania (1983-1991) NTLP report 1992.

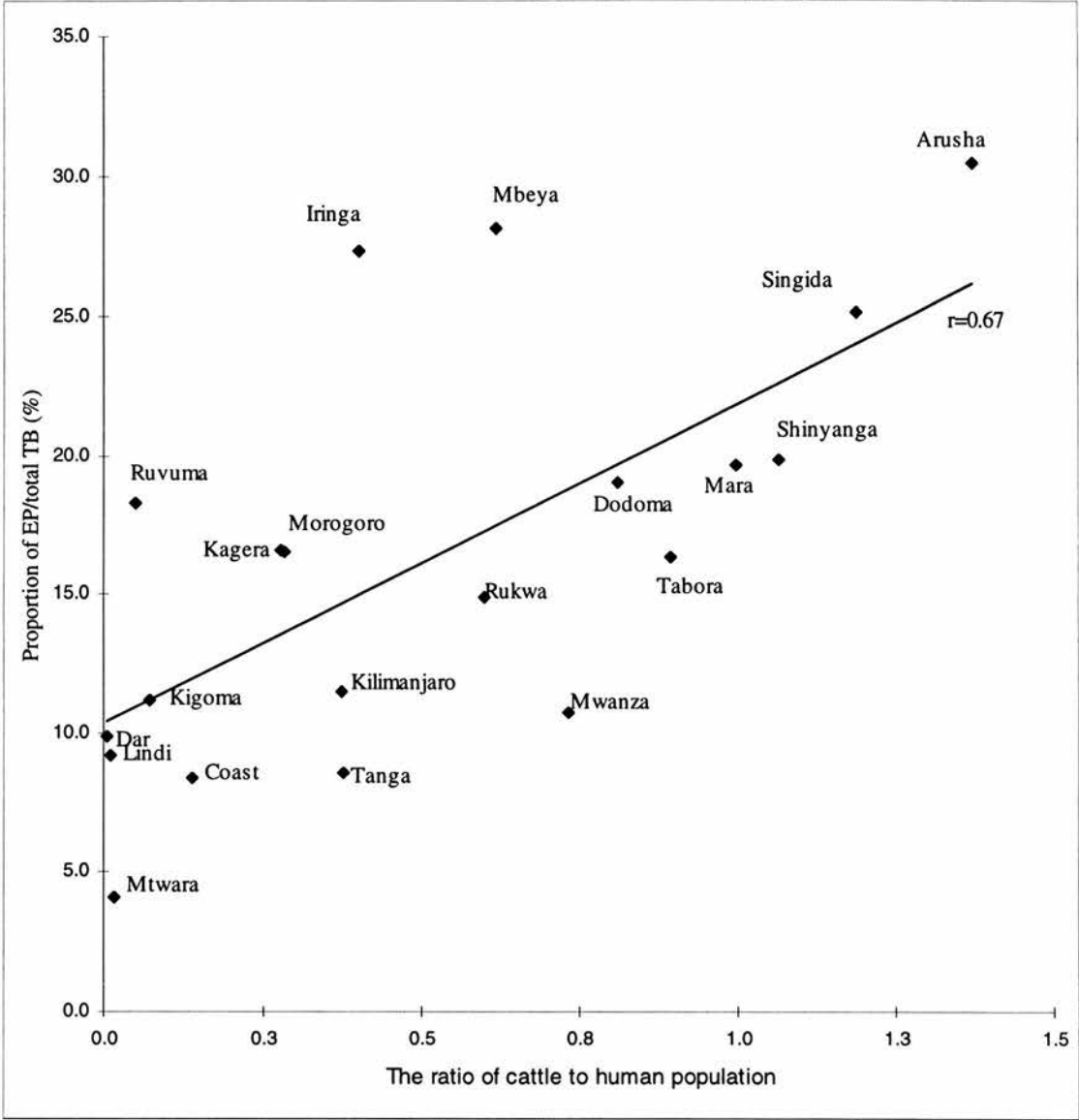


Figure 1.4. The relationship of cattle keeping and proportion of cases of extra-pulmonary tuberculosis to total tuberculosis cases (1990).

1.3. OBJECTIVES OF THE STUDY

In view of the above discussion, the objectives of this study, therefore were to:

- i) confirm the presence bovine tuberculosis in cattle by means of culture of specimens from abattoirs/slabs in the problem areas (Arusha, Mbeya and Iringa) of Tanzania,
- ii) confirm the presence of *M. bovis* infection in man by culture of specimens from human cases of tuberculosis in the problem areas of Tanzania,
- iii) study the mode of transmission of *M. bovis* from cattle to man in the pastoralist communities,
- iv) evaluate the existing molecular biology techniques for confirmation of the identity of strains of the *M. tuberculosis* complex and other mycobacteria from Tanzania,
- v) develop simplified *M. tuberculosis* complex typing techniques,
- vi) develop specific probes for the detection of *M. bovis*,
- vii) ascertain the genetic diversity of the strains of *M. bovis* from man and cattle in Tanzania

CHAPTER 2

GENERAL MATERIALS AND METHODS

CHAPTER 2 GENERAL MATERIALS AND METHODS

2.1. FIELD WORK

2.1.1. STUDY AREAS

The field work was carried out in 3 regions of Tanzania (*viz.* Iringa, Mbeya and Arusha), where previous data has indicated the presence of tuberculosis in cattle at slaughter and also where hospitals have recorded a high proportion of cases of extra-pulmonary tuberculosis in man.

Arusha region which is in Northern Tanzania (Figure 1.2), lies between 2°S and 5°S, and between 35°E and 38°E. The region has an average altitude of 1500m above sea level. Arusha region comprises seven administrative districts, namely; Arusha, Monduli, Mbulu, Hanang, Kiteto, Ngorongoro, and Arumeru. The major ethnic groups found in this region include, Maasai, Iraqw, Barbaigs, and Meru. The main occupations in this region include crop farming and livestock keeping.

Iringa and Mbeya regions form part of the Southern Highlands zone of Tanzania (altitude over 1500m above sea level). These regions are situated between 7°S and 11°S, and between 32°E and 37°E. Mbeya is made up of the following administrative districts; Mbeya (include Mbarali Sub-district), Chunya, Rungwe, Ileje, Mbozi, and Kyela. The districts making Iringa region are; Iringa, Mufindi, Njombe, Ludewa, and Makete. Within the Mbeya and Iringa regions are the Usangu Plains which are bordered by the east and west arms of the Rift Valley. Ruaha river divides the plains into north and south, and in the north of the plains is the Ruaha National Park. The major ethnic groups inhabiting the Mbeya region are, Nyakyusa, Safwa, Nyamwanga and Sangu, while in the Iringa region the major tribes are; Hehe, Bena, and Kinga.

The main occupation of people in the Southern Highlands is crop farming and livestock keeping, which is mainly confined to the vast areas of the Usangu Plains,

the valleys of the Ruaha river and Lake Rukwa basin in the west. There is substantial migration of cattle in between the Usangu Plains and the neighbouring regions.

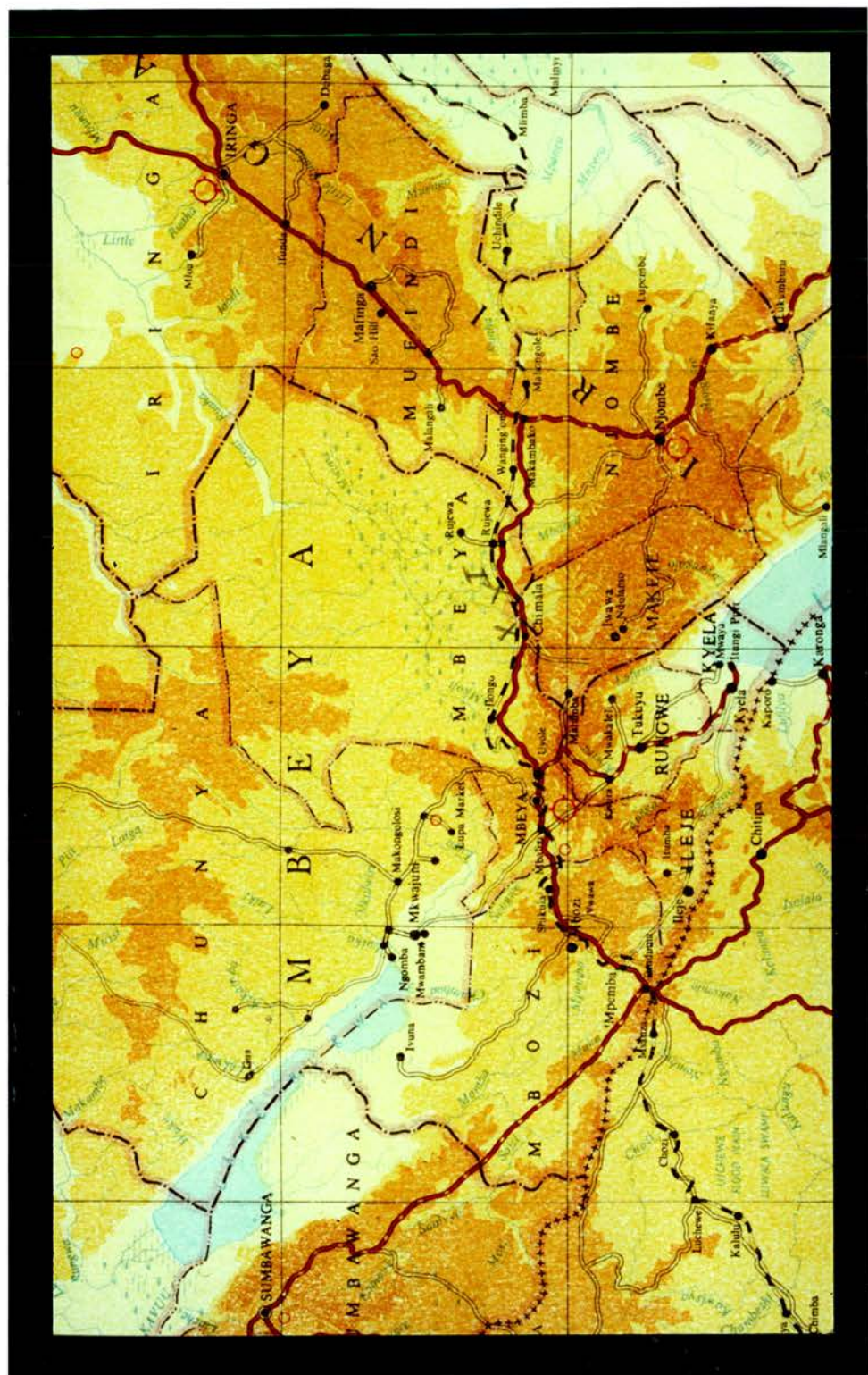
During the current study, slaughter slabs positioned in the plains were utilised to collect specimens from cattle. The selected slabs were situated across the plains from west to east as follows; Uyole, Igurusi, Chimala, Rujewa, Makambako and in the south east Njombe slab (Figure 2.1). Concurrently, hospitals and health centres serving the people of the Usangu Plains were utilised to collect human specimens. In the middle of the Usangu Plains was Matebete village. This village, exclusively inhabited by the Maasai pastoralists was included in an intensive study of the zoonotic implication of *M. bovis* in man and cattle.

2.1.2. STUDY POPULATIONS

2.1.2.1 Arusha region

2.1.2.1.1. Cattle

Cattle, destined for slaughter at the Arusha municipal abattoir, were included in this study. These cattle belonged to the major butchers in Arusha town. On average, the abattoir was processing 120 cattle per day. Some of the cattle, from which the specimens were collected, had been tuberculin tested earlier as part of a tuberculin test validation trial carried out under the auspices of the Bovine Tuberculosis (BTB) Project, of which the findings of the current study are part. The selection of cattle for this study was made arbitrarily but of necessity was biased by the co-operation of the cattle owners. These cattle originated from various livestock markets within and also outside the region and, in most of the cases, were trekked straight to the abattoir without a rest. All cattle, from which specimens were collected, had a project identification ear tag.



2.1.2.1.2. Human

Sixteen human cases of extra-pulmonary tuberculosis (particularly cervical lymphadenitis) encountered in the Mbulu and Hanang district were included in the study of the zoonotic importance of *M. bovis*. The two districts ranked highest in the region for having a high proportion of extra-pulmonary tuberculosis cases encountered from the mid 1980's to 1990 (NTLP report 1992).

2.1.2.2 Southern Highlands

2.1.2.2.1. Cattle

There were three sets of cattle involved in the current study. The first set was made up of 841 cattle destined for slaughter at seven slaughter slabs across the Usangu Plains from which the majority of lymph nodes were collected. These animals were earlier tuberculin tested by the author and members of the Edinburgh Veterinary Research Expedition Team -1994 (EVRET-94). EVRET-94 performed the study on validation of the tuberculin test in herds of cattle on the Usangu Plains. The second set comprised 4933 cattle, which were kept by pastoralists in the plains for various purposes. This set, apart from being tuberculin tested, had 871 milk samples collected from milking cows for the purpose of assessing the excretion of *M. bovis* through milk. The last set consisted of 652 cattle belonging to pastoralists of Matebete Village where, it was claimed that the number of human cases of tuberculosis had been high for many years (Mr. Tera Paraboi - Matebete Village Chairman - personal communication 1994). These cattle were tuberculin tested and milk samples collected from cows.

2.1.2.2.2. man

In order to ascertain the zoonotic importance of *M. bovis* in the Southern Highlands, cases of tuberculosis reported by the health centres adjacent to the selected slaughter slabs were included in the study. A total of 61 cases were identified by the District Tuberculosis and Leprosy Co-ordinator (DTLC) at Rujewa, and his subordinates stationed at health centres in Chimala and Igurusi. An additional 15 cases were identified by the Regional Tuberculosis and Leprosy Co-ordinator at Mbeya

Hospital, these were cases referred for surgery to remove enlarged cervical lymph glands. Surgeons at Ilembula Hospital also were asked to participate in identifying and performing operations on cases of extra-pulmonary tuberculosis from patients reporting at this hospital. A total of 8 cervical lymphadenitis cases had their glands removed at this hospital. This hospital, which is situated on the eastern side of the plains received many patients from the Usangu Plains. Field visits also were made by the DTLC to villages far away from the health centres in order to obtain specimens (mainly sputum) from pastoralists who due to transportation problems were unable to attend tuberculosis clinics at health centres and hospitals. A total of 39 cases were encountered during such visits.

2.1.2.3. Dar-es Salaam and Morogoro

Additional human samples were sought from Muhimbili Medical Centre (MMC), Dar-es-Salaam and Morogoro Regional Hospital. 32 cases of extra-pulmonary tuberculosis encountered at routine tuberculosis clinics and at emergency laparotomy. These samples were intended to provide a comparison group for the rural study as majority of cases referred to MMC and Morogoro Regional Hospital are either of town/city dwellers or referred from other regional hospitals in Tanzania.

2.1.3. COLLECTION OF SPECIMENS

2.1.3.1. Cattle

2.1.3.1.1. *Lymph nodes*

The major lymph nodes of the respiratory and alimentary system were collected from slaughtered cattle at routine gross inspection. The lymph nodes involved were retropharyngeal, submandibular, bronchial, mediastinal, and mesenteric lymph nodes. These lymph nodes were collected aseptically by meat inspectors, members of EVRET-94 and the author at all the slabs in the Usangu Plains. Specimens from cattle slaughtered in Arusha abattoir were kindly collected by the meat inspectors and a specialist working with the BTB Project in Arusha. A total of 841 and 1068 pooled

lymph nodes were collected at slaughter slabs in the Usangu Plains and the municipal abattoir in Arusha.

The collected lymph nodes, were subjected to 2mm slicing to ascertain the presence of additional minute lesions. The pooled lymph nodes from each animal were thereafter placed in either a 25 ml screw capped plastic universal container or in a snap-seal bag. The containers were labelled properly with the tag number of the respective animals, and then stored in the freezers at -20°C prior to shipment in ice-cold cool boxes to the Mycobacterium laboratory at Sokoine University of Agriculture (SUA), Morogoro,.

2.1.3.1.2. Milk samples

Collection of milk samples from cattle belonging to the pastoralists in the Usangu Plains was carried out by owners of the cattle in the early morning. Properly labelled screw-capped universal containers, accompanied with instructions for collection of milk were provided by the author or members of EVRET 94 to cattle owners the evening prior to milking. Samples were collected and then placed into cool boxes and taken to nearby refrigerator/freezers for storage in a similar manner to lymph nodes.

2.1.3.1.3. Nasal secretion

Attempts were made to extract nasal secretions from the nostrils of cattle belonging to pastoralists of Matebete village. A vacuum suction pump attached to a long plastic tubing was applied intranasally. This exercise was abandoned after a few attempts due to problems of restraining the cattle, and the dusty or muddy conditions which were created as a result of the milling cattle and people during catching and restraint. In most cases the collection tube was full of sludge made of mucus, faecal material and soil particles.

2.1.3.2. man

2.1.3.2.1. Lymph nodes

Lymph nodes were obtained only from hospitals. The removal of affected glands from individual patients was the responsibility of the surgeons. Screw-capped plastic

universal containers were used in collection. The collected lymph nodes were stored at -20°C before being conveyed in ice-cooled boxes to the Mycobacterium laboratory at SUA.

2.1.3.2.2. *Sputum samples*

The bulk of the sputum samples were collected at the health centres situated in the middle of Usangu Plains, where there were no facilities for performing surgery on cases of extra-pulmonary tuberculosis. Sputum was collected as described in the NTLP manual (NTLP 1987). These samples were collected in wide-mouthed plastic containers, properly identified, then stored in refrigerators or freezers at the health centres prior to transport in ice-cooled boxes to the specialist laboratory at SUA.

2.1.4. TUBERCULIN TESTING OF CATTLE

The single comparative intradermal tuberculin (SCIT) test (Lesslie and Herbert 1975) was used to test all cattle involved in this study. The tuberculin was a kind gift of two laboratories, namely the Central Veterinary Laboratory, Weybridge, Surrey, U.K. and the Central Veterinary Laboratory, Lelystad, The Netherlands. Both laboratories made their avian and bovine PPD according to European Union standards. They used strain AN5 and D4ER to produce bovine and avian PPDs, respectively. In the course of the present study, 0.1mg of bovine PPD and 0.05mg of avian PPD were injected intradermally at two shaved spots, 12cm apart, on one side of the neck. The blue and red M^cLintoch 2ml automatic syringes (M^cLintoch - Glasgow, UK) were used to inject bovine and avian tuberculins, respectively. All tuberculin tested cattle were provided with an identification ear tag, and records pertaining to the animals were recorded using the format indicated in Appendix I.

2.1.5. EPIDEMIOLOGICAL DATA COLLECTION AND ANALYSIS

2.1.5.1. Cattle

Epidemiological data in respect of each animal slaughtered/ tuberculin tested was collected by the members of EVRET-94 by filling the questionnaire designed by the author and members of EVRET-94. (Appendix I). The questionnaire was designed to obtain particular biodata of the animals. It was impossible to obtain extensive information on the source of the animals. Most of cattle destined for slaughter were obtained from local livestock markets, and might have changed ownership several times prior to slaughter. It was, however, known that the majority of cattle slaughtered in the slabs were from within the Usangu Plains, with exception of the slabs on the periphery of the plains. There was no similar questionnaire for the Arusha study, as this part of the project started prior to formulation of the questionnaire.

The questionnaire was designed using EPINFO version 6 software (Centre for Disease Control, Epidemiology Program Office, Atlanta, Georgia, USA/World Health Organisation, Global Programme on AIDS, Geneva, Switzerland), therefore all the statistical analysis of the data collected from individual animals was analysed using the same software package.

2.1.5.2. Human cases

The questionnaire for collection of epidemiological data of all human cases encountered also was designed using EPINFO version 6 software. There were two types of questionnaire, a detailed and brief one (Appendix II&III). Although medical surgeons were encouraged to use the detailed questionnaire, it was sometimes not possible as they were occupied with their daily routine, and hence either did not use the form or filled only parts of it. However, fully filled forms were obtained from almost all cases encountered in the rural health centres. As with the previous questionnaire, all the statistical analysis was performed using the same software.

2.2. LABORATORY WORK

2.2.1. BACTERIOLOGY

2.2.1.1. Sokoine University of Agriculture (SUA) - Mycobacterium Laboratory

2.2.1.1.1. Sample processing

Samples received from the field were stored in freezers at -20°C up to the time of processing.

All the processing of specimens from cattle and man was carried in the Class one safety cabinet (Medical Air Technology - Manchester, U.K).

Pool of lymph nodes were taken from containers aseptically using sterilised forceps and placed in sterile containers. Using sterile scalpel blades, samples were further macerated to obtain fine pieces which were divided into two portions. One portion was stored at -20°C for future reference, while the other portion of chopped lymph nodes was placed in the stomacher bag containing about 5ml of distilled water and homogenised for five minutes using a Stomacher 80 Lab blender (Seward Laboratory - London, UK). The lymph node homogenate was then put into a universal container and 3% oxalic acid was added to fill the universal for decontamination. Decontamination was carried out for 30 minutes with intermittent shaking. The duration of the decontamination step was increased if contamination in the specimens was a problem.

Regarding sputum and milk samples, half of the sample was transferred into a 25 ml universal container, and was decontaminated using 4% sodium hydroxide (NaOH). Homogenisation was achieved within 15 minutes.

After the allotted decontamination time, the lymph node homogenate was centrifuged at 1500g for 20 minutes and the supernatant discarded into a disinfectant. 2% NaOH was added to neutralise the pH of the samples. In the case of the sputum and milk samples, an indicator; phenol red was added prior to neutralisation using concentrated HCl. Neutralisation was achieved when the suspension colour changed from purple to pink. Suspensions were then centrifuged and the supernatant discarded to leave at least 2 ml of the sediment to be used as inoculum for the cultivation of mycobacteria.

2.2.1.1.2. Microscopy

A loopful of suspension from the above procedure was spread on a glass slide and heat fixed within the safety cabinet. Glass slides were then placed on a rack over a sink for ZN staining in a similar manner to that described by Grange (1988). The observation of acid-fast-bacilli (AFB) was carried out at X100 magnification and results recorded.

2.2.1.1.3. Culture

2.2.1.1.3.1. Media preparation

Instruction for the composition and preparation of media were provided by Scottish Mycobacterium Reference Laboratory (SMRL), City Hospital, Edinburgh.

For primary isolation of mycobacteria, the egg media namely; IUT and Loewenstein-Jensen with added pyruvate (L-J pyruvate) were used. The media compositions were as follows: i) IUT medium was made up of 61.0% whole egg, 36.6% (v/v) IUT buffer salt solution (50mM K_2PO_4 ; 25mM $Na_2HPO_4 \cdot 2H_2O$; 1.6mM $MgSO_4 \cdot 7H_2O$; 14mM citric acid; 67mM L-Asparagine; 0.2% glycerol) and 2.4% (v/v) of 1% (w/v) malachite green, and ii) L-J pyruvate medium was made of 64.6% whole egg, 32.3% (v/v) pyruvate medium buffer salt solution (50mM KH_2PO_4 ; 25mM $Na_2HPO_4 \cdot 2H_2O$; 114mM sodium pyruvate; 14mM Citric acid), 2.6% (v/v) of 1% (w/v) malachite green and 0.6% (v/v) of 1% (w/v) trypan blue.

After mixing all the components, 4 ml of the medium was dispensed into 30ml glass universals and then inspissated at 85°C for one hour to solidify. IUT medium had a pale green colour, while L-J pyruvate medium was blue.

2.2.1.1.3.2. Cultivation of *Mycobacterium*

About 0.1ml of the sediments from each sample was spread on the surface of each of the media using a sterile pastette and, in order to avoid sedimentation of inoculum at the bottom of the slope, all the slopes were laid horizontally overnight before being placed vertical for continued incubation. Cultures were incubated at 37°C for at least six weeks, with weekly observation for signs of growth. For cultures suspected to be contaminated, stored sediments were re-treated for a longer duration and streaked

onto a blood agar plate. Blood agar plates were incubated overnight, and if signs of contamination persisted, the sample was treated further by doubling the decontamination time. The positive cultures were those that provided colony morphology similar to that described by Vestal and Kubica (1966).

2.2.1.1.4. Identification

Positive cultures were subsequently subcultured onto another set of media and incubated for another three to four weeks.

The first step towards species identification was the visual observation of growth on IUT and L-J pyruvate media. According to Marks (1976), *M. tuberculosis* produces eugonic growth on both media, while *M. bovis* only grows well on L-J pyruvate medium. Colonies were stained with ZN as described above and examined for AFB. The presence of AFB and cording of bacilli were indicative of presence of mycobacteria species.

Due to a limitation of resources, the SUA Mycobacterium laboratory was able to perform fewer additional identification tests. These were; i) growth at 37°C and 45°C, to differentiate *M. tuberculosis* complex from MOTTs, particularly the *M. avium* group, ii) growth on medium containing para-nitro benzoic acid (PNB), the medium supporting growth of MOTTs and not *M. tuberculosis* complex, and iii) niacin test performed according to manufacturers instruction (TB Niacin Test Reagent - DIFCO Laboratories, Detroit, Michigan, USA). In the niacin test *M. tuberculosis* gives a positive reaction while *M. bovis* is negative.

Confirmed mycobacteria strains were sub-cultured on up to 4 slopes each of IUT and L-J pyruvate medium to obtain enough cells for molecular analysis. Each strain was sent to SMRL, Edinburgh, for confirmation of identity and passed on to the Moredun Research Institute (MRI) Edinburgh, UK, for further molecular biology tests.

2.2.1.2. Scottish Mycobacterium Reference Laboratory (SMRL)

Cultures of strains from Tanzania, were sent to SMRL for two reasons: i) to confirm the identity of strains, which also served as a quality control measure for the performance of the SUA Mycobacterium laboratory, and ii) the Scottish laws

regarding the importation of live pathogens required that all strains were received by a specialist laboratory for verification of identity before passing on to less specialised laboratories. A number of tests were used by SMRL to identify species of *Mycobacterium tuberculosis* complex (Watt *et al.*, 1993). After confirming the identity of strains, pure cultures were taken to the Mycobacterium laboratory at MRI.

2.2.1.3. Mycobacterium Laboratory at MRI

The bacteriological work at this laboratory, was carried out in accordance with training received at SMRL.

At MRI cultures were sub-cultured onto either duplicate slopes of IUT and L-J pyruvate or into Middlebrook 7H9 broth (DIFCO Laboratories - Detroit, USA) with ADC supplement (DIFCO Laboratories - Detroit, USA) as described by the manufacturers. Preparation of media and incubation conditions were similar to those described above. The growth of mycobacteria was monitored once every week. Decontamination was carried out in a similar manner to one used by SUA laboratory in case of contamination of slopes. Particular care was taken in monitoring contamination of the broths with regular checks by ZN and Gram stained smears.

When enough colonies have grown on the surface of the solid media, these were harvested by scraping, using sterile disposable plastic loops. The harvested cells were placed in a sterile universal bottle containing 20ml of distilled water and mixed thoroughly. The sealed universals containing harvested cell were placed in a water bath at 70°C for at least 30 minutes in order to kill the cells. In the case of liquid medium, the entire contents of the tube were treated in the same manner to cells from solid media. After heat treatment, cells were pelleted by centrifugation at 2500g at 4°C for 10 minutes. The pellets were weighed, recorded and stored at -20°C until used.

In order to maintain the strains, fresh sub-cultures were made from original slopes onto one of the egg media and when enough colonies were visible, a few colonies were picked and placed in a bijoux containing 2 ml of Dubos broth, made as recommended by the SMRL instruction manual. The stock strains were then stored frozen at -20°C.

2.2.2. MOLECULAR BIOLOGY TECHNIQUES

All strains of mycobacteria, particularly members of *M. tuberculosis* complex, were subjected to molecular biology analysis using polymerase chain reaction (PCR) for confirmation of their identity and restriction fragment length polymorphism (RFLP) and spoligotyping for DNA fingerprinting to determine their genetic relatedness.

2.2.2.1. DNA extraction

The first step towards carrying out any of the molecular biology tests was a successful extraction of DNA from cultured mycobacteria.

The extraction of DNA was carried out by Mycobacterium laboratories of SUA and MRI. Methods used to extract DNA from harvested cells were either by enzymatic digestion of cells, by mechanical breaking of cells using a bead beater or by boiling of cells to release the DNA.

2.2.2.1.1. Enzymatic DNA extraction

The protocol used for this exercise was a modification of method described by Whipple *et al.* (1987). The amount and concentration of reagents were used on the basis of extracting DNA from 100 mg of wet weight of cells. DNA therefore was extracted as follows. Harvested cells were washed twice in 5ml TEN (50mM Tris-HCl pH8.0; 100 mM EDTA; 150 mM NaCl), the pellet transferred into a 1.5 ml eppendorf containing 500µl TE (10mM Tris-HCl pH8.0; 1mM EDTA) and mixed thoroughly. 8000 U of lipase (Sigma Chemicals - St Louis, USA) were added and incubated for 2 hours at 37°C. 250µl of 10 mg/ml lysozyme (Sigma Chemicals - St Louis, USA) was added and incubated at 37°C for 2 hours before the addition of 75µl each of 20mg/ml proteinase K (Sigma Chemicals - St Louis, USA) and 10% lauryl sulphate (SDS). The contents were then incubated for 16 hours at 50°C. After incubation, 360µl of cold 5M potassium acetate (KOAc) (60ml 5M KOAc; 11.5ml glacial acetic acid; 28.5ml distilled water) was added, mixed well and placed on ice for 10 minutes, followed by centrifugation at 13,500g for 10 minutes. 750µl of the

supernatant was transferred to a clean eppendorf and equal volume of phenol (water saturated) was added, mixed by inversion and centrifuged as above for 5 minutes. The top aqueous layer was transferred to a clean eppendorf, and an equal amount of phenol/chloroform and isoamyl alcohol (24:1v/v) was added, mixed and centrifuged as above. The top layer was transferred to a clean eppendorf and equal volume of chloroform:isoamyl alcohol (24:1v/v) added, mixed and centrifuged as above. The top aqueous layer was transferred to clean eppendorf and DNA precipitated by adding a 1/10th volume of 3M sodium acetate (NaOAc) pH5.2 and 2 volumes of ethanol and mixing well. The contents were then kept at -20°C for at least six hours prior to centrifugation at 13,500g for 30 minutes. The liquid was carefully discarded and the DNA pellet washed in 70% (v/v) ethanol. The pellet of DNA was air dried and then re-suspended with 50µl of distilled water and stored at either 4°C or -20°C ready for subsequent use.

2.2.2.1.2. Mechanical DNA extraction

This procedure was carried out as an alternative rapid method to obtain DNA for PCR procedures which did not require good quality DNA for amplification of product.

2.2.2.1.2.1. Extraction of DNA by bead beating

The procedure was performed in a similar manner to the published protocol (Challans *et al.*, 1994). 0.1mm zirconium beads (Biospec Products - Oklahoma, USA) were used to break the cells in order to extract DNA. An ESPE Capmix® micro-bead beater (ESPE GmbH - Germany) was used in this exercise. In brief, a 2ml screw capped vial was filled to half its volume with zirconium beads and a cell pellet (~ 10-20mg), re-suspended in 500µl NaOH, was added and shaken on the micro-bead beater at high speed for 3 minutes (2X1.5 minute stages with cooling on ice for 30 seconds in between). The beads and disrupted cell debris pellet was compressed by centrifugation at 6500g and the supernatant removed and placed in a clean eppendorf for DNA recovery. In order to extract DNA, 450µl of GE (5M guanidine isothiocyanate; 0.1M EDTA) was added to the eppendorf, followed by the addition of

250µl of ice cold 7.5M ammonium acetate and incubation on ice for at least 15 minutes. After incubation, 500µl of chloroform/octanol (24:1v/v) was added and centrifuged at 13000g for 5 minutes. The top aqueous layer was transferred to a clean eppendorf and the previous step repeated. The top layer was again taken to a clean eppendorf and an equal volume of isopropanol added and mixed by inversion before being centrifuged at 13000g for 5 minutes. The supernatant was poured off and the pellet washed twice with 70% ethanol. The DNA pellet was then air dried and re-suspended in a similar manner to above.

2.2.2.1.2.2. Extraction of DNA by boiling of cells

Another simpler procedure for extraction of DNA used in this study was by boiling of cells suspended in 25 µl of distilled water for 15 minutes and then centrifuging at 13000g. 1µl of the supernatant was then used for PCR procedures.

2.2.2.2. Determination of quality and quantity of DNA

The quality and quantity of the DNA extracted was determined by visual comparison of the intensity and conformation of DNA bands of template DNA with those of different concentrations of Lambda (λ) DNA (Pharmacia Biotech - Uppsala, Sweden) on ethidium bromide stained 0.8% agarose gel. A total of 2-3µg of DNA was required for procedures such as Southern blotting and subsequent restriction fragment length polymorphism (RFLP) analysis, whereas most PCR procedures required 50ng (or less) of DNA. A good DNA was that produced a compact band with little smearing below the loading well.

2.2.2.3. Molecular identification - Polymerase chain reaction (PCR)

The general equipment and conditions used for PCR procedures during the course of this study were as follows;

2.2.2.3.1. Equipment

The PCR machines used were the Techne PHC-3 (Techne - Cambridge, UK) at MRI and the Crocodile II (Appligene Inc. - Pleasanton CA, USA) at SUA.

2.2.2.3.2. *General conditions for PCR*

Manipulation at different stages of performing PCR were carried out in four different areas, well separated from each other. DNA preparation was made in Mycobacterium Laboratory, reagents for PCR were kept and mixed in a separate room, and the PCR machine was kept in a third room far apart from amplification analysis lab. These precautions were intended to prevent contamination of reagents with DNA and amplicons.

The first step in setting up the PCR was to standardise the concentration of DNA by making dilutions of the stock DNA. Most PCRs required at least 50ng of template DNA to generate the desired products.

For a standard PCR, the following reagents were necessary to make the master mix; all four of deoxynucleotide triphosphates (dNTP's) (Pharmacia, Biotech. - USA), forward and reverse primers (Oswel DNA Services, Edinburgh/Southampton, UK), 10X PCR reaction buffer (50mM NaCl/KCl; 1.0-2.5mM MgCl₂; 10mM Tris-HCl; 0.01% gelatin), and *Taq* DNA polymerase (Boehringer Mannheim - Germany). Denaturation of DNA was achieved by placing an eppendorf with diluted template DNA (25-50ng/μl) into boiling water in a beaker for 10 minutes before quenching on ice and subsequently adding the desired volume to the master mix to make a final volume of 50μl for standard PCR. The reaction mix in a 0.5 eppendorf (Anachem UK) was overlaid with 50μl of mineral oil (Sigma Chemical co. - St Louis, USA).

A PCR cycle is comprised of denaturation, annealing and extension steps. For all standard PCRs, denaturation of DNA was carried out at 94°C. However, the annealing temperature varied according to the G+C and A+T sequences of the primers. A simplified formula for obtaining an appropriate annealing temperature (Thomas 1980).

$$\{(4(G+C) + 2(A+T)) - 5\} = \text{annealing temp.}$$

The primer extension was normally set at 72°C for most of the PCR protocols.

2.2.2.3.3. Conditions for *M. tuberculosis* complex specific PCRs

2.2.2.3.3.1. IS986 PCR

The method of Hermans *et al.* (1990) was used to amplify a 245bp product from within IS986. The PCR master mix was prepared as follows: 50mM NaCl, 2mM MgCl₂, 10mM Tris-HCl (pH8.3), 0.2mM dNTP's, 0.01% gelatin, 0.5 units (U) of *Taq* DNA polymerase and 500ng of each of the primers, designated B971 and B972 whose sequence and position within IS986 are presented in Figure 2.2 below. The amount of denatured template DNA added to the master mix was 100ng. A total of 30 PCR cycles were performed, each PCR cycle comprised of the following steps: denaturation at 94°C for 1 minute, annealing at 62°C for 1 minute and extension at 72°C for 2 minutes.

2.2.2.3.3.2. IS1081 PCR

The protocol for amplification of IS1081 was a modification of the method described by Collins *et al.* (1993). Two new primer sequences were used to produce a 248bp product rather than 298bp by primers published by Collins *et al.* (1993). The sequence and position of primers, designated G2008 and G2009, are presented in Figure 2.2. The PCR master mix was made up of the following: 50mM NaCl, 1.5mM MgCl₂, 10mM Tris-HCl (pH8.3), 0.2mM dNTP's, 0.01% gelatin, 0.5 units (U) of *Taq* DNA polymerase and 500ng of each of the primers. 100ng of template DNA was added to the master mix after initial denaturation. A total of 30 PCR cycles were performed, each PCR cycle comprised of the following steps: denaturation at 94°C for 1 minute, annealing at 68°C for 1 minute and extension at 72°C for 2 minutes.

2.2.2.3.3.3. *mtp40* PCR

This PCR was carried out in a similar manner to that described by del Portillo *et al.* (1991). The master mix was made up of the following; 1X PCR reaction buffer diluted from 10X PCR reaction buffer (100mM Tris-HCl, 15mM MgCl₂, 500mM KCl) (Boehringer Mannheim GmbH - Germany), 0.2mM dNTP's, 20pmol of each primer and 2.5U *Taq* DNA polymerase. 100ng of template DNA was added to the

master mix after initial denaturation. The primers, designated D315 and D316 (Figure 2.2) amplified a 396bp product from a gene encoding a protein, *mtp40* that is found only in *M. tuberculosis*. A total of 30 PCR cycles were performed, each PCR cycle comprised of the following steps: denaturation at 94°C for 1 minute, and annealing and extension at 74°C for 2 minutes.

2.2.2.3.3.4. MPB70 PCR

The protocol for MPB70 PCR as described by Wilton and Cousins (1992), was used to amplify a 372bp product from the gene encoding a major secretory protein (MPB70) of *M. bovis* (Wilton and Cousins 1992). The sequence and position of primers, designated G2965 and G2966, are presented in Figure 2.2. The master mix was made up of the following: 1X PCR reaction buffer (Boehringer Mannheim, GmbH Germany), 1mM dNTP's, 0.2mM of each of the primers and 0.5U *Taq DNA* polymerase. A total of 100ng of denatured template DNA was added to the master mix to make the final PCR reaction mix. A total of 30 PCR cycles were performed, each PCR cycle comprised of the following steps: denaturation 94°C for 1 minute, annealing 62°C for 1 minute and extension 72°C for 2 minutes.

2.2.2.3.3.5. IS986/*mtp40* Multiplex PCR

In order to identify species of the *M. tuberculosis* complex by PCR, Sinclair *et al.* (1995) developed a multiplex PCR which was able to discriminate strains of *M. tuberculosis* from the rest in the tuberculosis complex. The same primers to those used in the individual PCR for IS986 and *mtp40* were used. The PCR master mix was made of ; 50mM KCl, 2.5mM MgCl₂, 10mM Tris-HCl (pH8.3), 0.1mM dNTP's, 0.01% gelatin, 0.5 U of *Taq DNA* polymerase, 0.05µM of each of the primers for IS986, and 0.1µM of each of the primers for *mtp40*. A total of 30 PCR cycles were performed, each cycle was comprising of; denaturation at 94°C for 30 seconds, annealing at 62°C for 30 seconds and extension at 72°C for 2 minutes. Two PCR products at 245bp and 396bp were expected in strains of *M. tuberculosis*, whereas for *M. bovis* and other strains of the tuberculosis complex, a single product at 245bp was expected.

2.2.2.3.3.6. *M. bovis* specific PCR

The *M. bovis* specific PCR protocol published recently by Rodriguez *et al.* (1995) was included in this study for verification of its specificity to strains of the *M. tuberculosis* complex from Tanzania. The protocol was done in the same manner to the publication. Briefly, 50ng of denatured DNA was mixed with a master mix made of 1X reaction buffer (Boehringer), 75pmol of each of the primers T2175 (JB21) and T2176 (JB22), 0.2mM dNTPs, and 2.5U *Taq* DNA polymerase. The reaction was subjected to 30 cycles of amplification, with each cycle having a denaturation step at 94°C for 1 minute, an annealing step at 68°C for 30 sec, and extension step at 72°C for one minute. This PCR was reported to amplify a product of 495bp in *M. bovis* only (Rodriguez *et al.*, 1995).

2.2.2.3.4. Analysis of the PCR products

Regarding visualisation of PCR products, this was either by agarose or polyacrylamide gel electrophoresis. Before loading PCR product into the wells of the gels, a 1/5 volume of loading buffer (10% Ficoll 400; 75mM EDTA; 0.1% xylene cyanol FF; 0.1% bromophenol blue) was added and mixed. For each analysis, one well on the gel was loaded with molecular weight marker - 1 KB ladder (Gibco-BRL - UK).

2.2.2.3.4.1. Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed according to Sambrook *et al.* (1989). A 7.5% gel was made of 30% (v/v) acrylamide (Scotlab - Scotland, UK), 5XTBE (445mM Tris-HCl; 12.5mM EDTA; 445mM boric acid), 0.1% (w/v) ammonium persulphate and 0.1% (v/v) N,N,N',N' - tetramethylethylenediamine (TEMED - Sigma Chemical Co. St Louis USA). Gels were cast using the Mini Protean II gel kit (Biorad USA). After the wells have been loaded with PCR products and molecular weight marker, electrophoresis was carried out at a constant 200V for 30 minutes using a vertical Mini Protean II electrophoresis apparatus (Biorad - U.S.A.) filled with 1XTBE buffer.

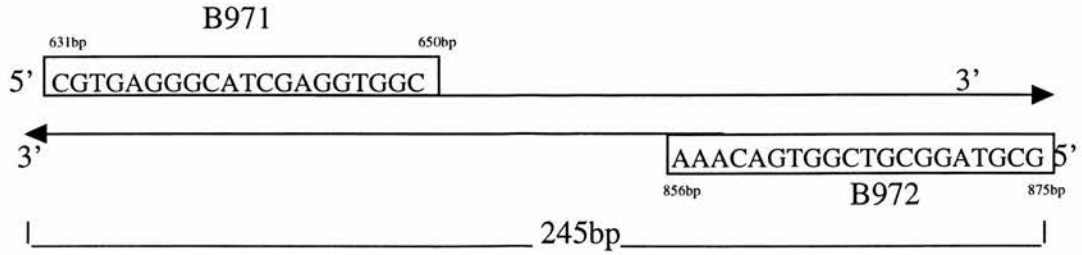
Polyacrylamides gel were stained by silver nitrate as described by Herring *et al.* (1982). The gels were, fixed initially in 100ml of 10% (v/v) ethanol and 0.5% (v/v) glacial acetic acid for 5 minutes with gentle agitation, followed with staining in a 0.19%(w/v) silver nitrate solution for five minutes. After two washings with distilled water, DNA fragments were visualised by agitating the gel in a solution of 3% (w/v) NaOH and 0.75% (v/v) formaldehyde for at least 6 minutes. The colour development was stopped using 0.75% (w/v) Na₂CO₃ solution. Gels were preserved in sealed polythene bags at 4°C.

2.2.2.3.4.2. Agarose gel electrophoresis

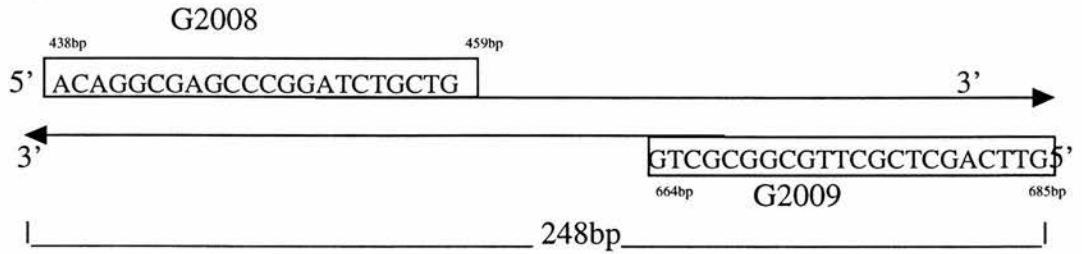
Agarose gels were made by mixing the required amount of agarose powder (Multipurpose agarose - Boehringer Mannheim GmbH-Germany) with 0.5XTBE to obtain the desired concentration of the gel. For analysis of larger fragments i.e. >1 kilo base pairs (Kb), the percentage of gel was lowered to 0.8% while for visualization of small fragments <1Kb, a 1 to 1.2% agarose gel was used. Agarose was dissolved by heating the solution in a microwave or on a hot plate. Five millilitre of 0.5µg/ml ethidium bromide solution was added to every 100ml of cooled molten agarose. Molten agarose was then poured into the electrophoresis gel casting equipment and left for approximately 15 minutes for the gel to set.

PCR products were loaded into wells and run in 0.5XTBE buffer in a horizontal gel electrophoresis apparatus at a constant voltage of 80V for 1½ hours, molecular weight marker were run in a parallel track. Visualisation of bands was made by medium wavelength ultra-violet (UV) light Transluminator UVP, (Ultra Violet Products - San Gabriel, CA, USA). Documentation of the PCR product bands was done using instant polaroid camera systems; viz DS-34 Camera system (Sigma-Aldrich Techware - Dorset, UK) at SUA, and a Polaroid MP-4 Land Camera 44.01(Polaroid - Japan) at MRI.

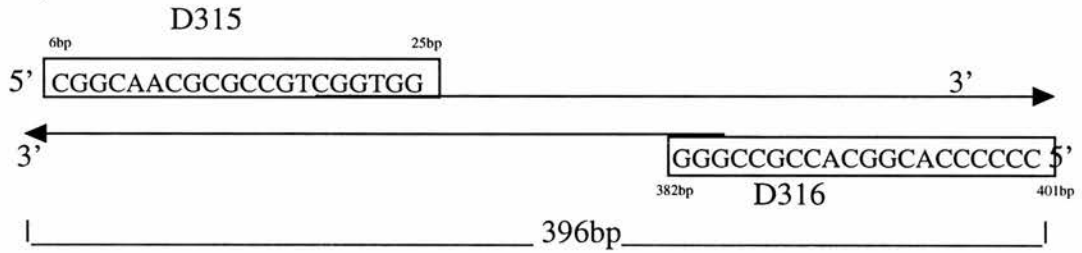
1. IS986 PCR



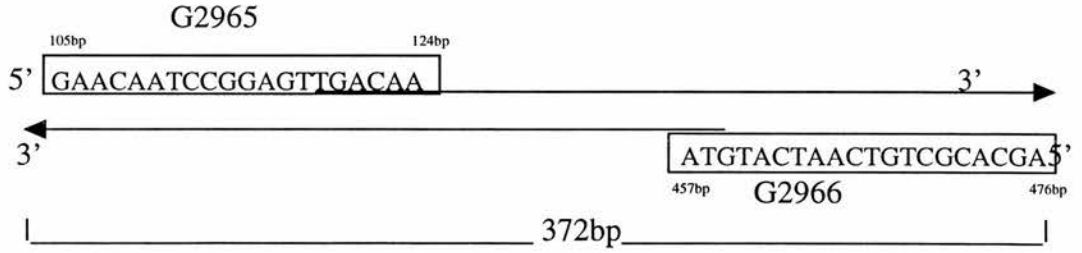
2. IS1081 PCR



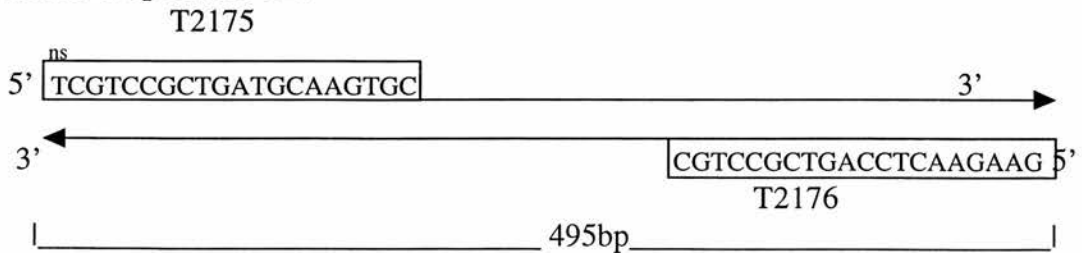
3. mtp40 PCR



4. MPB70 PCR



5. *M. bovis* specific PCR



ns Entire DNA sequence not published

Figure 2.2. Primers for various PCRs- primer sequence, position and size of product

2.2.2.4. Molecular typing - DNA Fingerprinting

2.2.2.4.1. Southern blot hybridization - Restriction fragment length polymorphism (RFLP)

In order to carry out RFLP DNA fingerprinting, the following major procedures were involved: i) Digestion of DNA and electrophoresis, ii) transfer of DNA from the gel to the membrane iii) choice and preparation of the probe iv) hybridisation of target DNA on the membrane, and v) detection and documentation of the hybridised products.

2.2.2.4.2. Digestion of DNA and electrophoresis of fragments

Throughout the course of this exercise, DNA was digested as described by van Embden *et al.* (1992). Briefly, the restriction endonuclease *PvuII* (CAG↓CTG) represented once within the IS986 (Figure 2.3a) and not present within pTBN12 (Figure 2.3b) was used to digest template DNA intended for DNA finger printing. The choice of *PvuII* for digestion was based on the fact that the 245bp fragment of DNA derived from IS986 PCR had no restriction site for *PvuII* and therefore would hybridise only once with template DNA containing IS986. Similarly, its absence in pTBN12 would result to a single hybridisation on genomic DNA *PvuII* cleaved fragment containing pTBN12 DNA sequence.

Digestion of DNA was carried out as follows. 2-3µg of good quality DNA was mixed in a 0.5ml eppendorf tube with 1X restriction buffer M (Boehringer Mannheim - Germany), 10U of *PvuII* (Boehringer Mannheim - Germany) and the required volume of distilled water to make up a desired volume. The digestion mixture was mixed thoroughly and centrifuged before incubating overnight in water bath at 37°C.

Digested DNA was separated by agarose gel electrophoresis as described above, except that a longer gel (20cm) was used, and the running voltage was set at 2 volts per cm (40V). This step was carried out for 20 hours with the electrophoresis buffer changed every 6 to 8 hours. Co-electrophoresis of 1Kb DNA ladder (Gibco-BRL - UK) was essential to estimate fragment sizes.

After electrophoresis, the gel was stained by gentle agitation in 0.5µg/l ethidium bromide solution for 15 minutes. DNA fragments were visualised under UV light,

and a illuminating ruler was placed at the side of the gel adjacent to the molecular weight markers to assist with approximation of the size of fragment on the membrane or autoradiograph. A photograph of the gel was taken as a permanent record of the band positions.

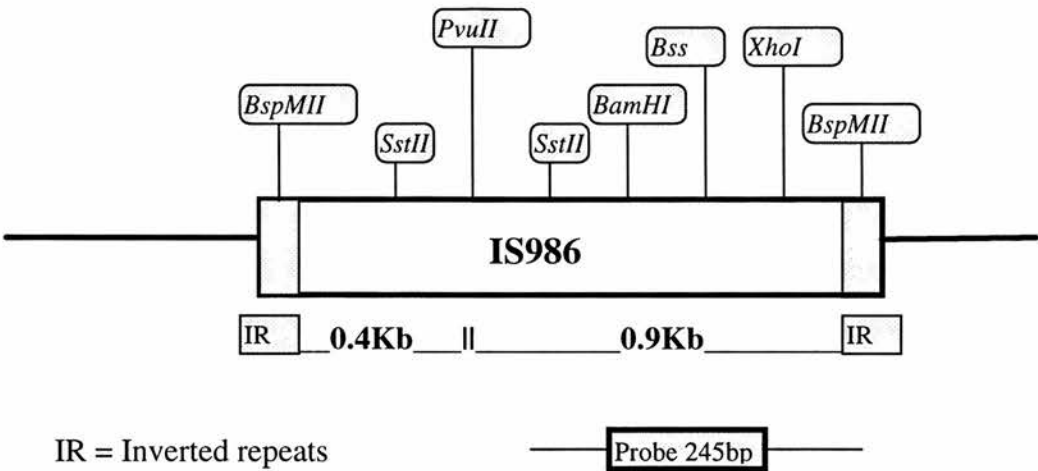


Figure 2.3a. The physical map of IS986 (1.35Kb)

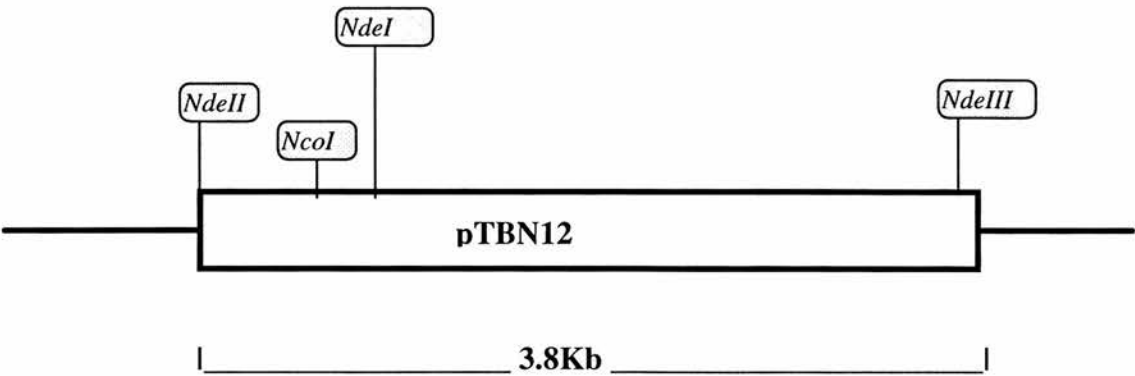


Figure 2.3b. The physical map of pTBN12 (3.8Kb)

2.2.2.4.3. Transfer of DNA fragments from gel to membrane (Southern Blotting).

The procedure for Southern blotting was a modification of the method of Southern (1975) and Sambrook *et al.* (1980). In brief, the agarose gel was washed twice in distilled water to remove excess ethidium bromide, then agitated for 30 minutes in denaturation solution (1.5M NaCl; 0.5M NaOH), washed in distilled water and agitated further in neutralisation solution (1.5M NaCl; 0.5M Tris-HC pH7.2; 1mM EDTA) for another 15 minutes.

DNA fragments were transferred to the membrane as follows. A tray was filled with 20XSSC (3M NaCl; 0.3M sodium citrate) blotting buffer and Whatmann 3MM blotting paper was cut so that it overhung two sides of the glass platform and dipped into the blotting buffer. The gel was then placed on the top of saturated blotting paper, taking care to avoid trapping air bubbles below the paper. A nylon membrane (HybondTM-N+- Amersham UK), cut to the size of the gel, was soaked in blotting buffer and then placed on top of the gel taking similar care to remove air bubbles trapped beneath the membrane by rolling a piece of 5ml pipette from side to side. A piece of cling film was placed on the exposed side of the gel to avoid transfer of the buffer directly to the stack of absorbent paper placed on the top. A 0.75 -1Kg weight was placed on top of the stack of paper. The blotting buffer passes through the gel drawn by the blotting paper and carries the DNA, which becomes trapped in the nylon membrane. The minimum time for transfer was 16 hours.

After transfer, the capillary was dismantled carefully. Using a pencil, the position of wells was marked for later identification. A two step DNA fixation was carried out. First the membrane (DNA side up) was placed for 30 minutes on a layer of 3MM Whatman paper saturated with 0.4M NaOH followed by baking in oven set at 80°C for 1 hour. Dried membranes were wrapped with cling film and stored at 4°C until used.

2.2.2.4.4. DNA probe preparation

In the course of this study, two types of DNA probes were used to type respectively strains of *M. tuberculosis* and *M. bovis*. The former was probed with a 245bp IS986 (van Embden *et al.*, 1993) PCR product, whereas the latter was probed with pTBN12

(Ross *et al.*, 1992). pTBN12 was excised from its vector by double digestion with *Hind III* and *EcoRI* (Boehringer Mannheim- Germany). The two DNA fragments were recovered from 0.8% low melting point (LMP) agarose (FMC Bioproducts - Rockland, USA) by either melting the gel followed by phenol/chloroform extraction of DNA as described above or by use of the QIAquick Gel Extraction kit (QIAGEN GmbH - Hilden, Germany) according to manufacturer's instruction manual. The labelling of DNA probes was done by a modification of a method described by Feinberg and Vogelstein (1983).

2.2.2.4.5. Non-radioactive labelling

For non-radioactive labelling, DNA probes were labelled with digoxigenin-dUTP (Boehringer Mannheim- Germany) by random oligonucleotide priming. Individual steps for labelling were carried out in accordance with the instructions provided by the manufacturer. In brief, the DNA was denatured fully by boiling at 100°C for 10 minutes, then mixed on ice with 2µl hexanucleotide mix (Boehringer Mannheim - Germany), 2µl dNTP mixture (1mM dATP; 1mM dGTP; 1mM dCTP; 0.65mM dTTP; 0.35mM DIG-dUTP; pH7.5) and 1µl Klenow enzyme. The mixture was vortexed and centrifuged prior to incubation in a water bath at 37°C for a period of 16 to 20 hours.

2.2.2.4.6. Radioactive labelling

Occasionally, radioactive DNA probe labelling was carried out in cases where the previous method did not offer adequate hybridization signal. In this procedure [$\alpha^{32}\text{P}$] dCTP (Amersham UK) was used as a label. Steps for labelling were done according to those described in the instruction manual for High Prime (Boehringer Mannheim - Germany). Briefly, 25ng (11µl) of denatured DNA was mixed with 4µl of High prime solution (200µl random primer mixture; 1unit Klenow polymerase, labelling grade, 0.125mM dATP, 0.125mM dGTP, 0.125mM dTTP, and 5X stabilising reaction buffer in 50% (v/v) glycerol) and 1.85MBq (5µl) [$\alpha^{32}\text{P}$] dCTP (Amersham - UK), and the mixture incubated at 37°C for 30 minutes.

The labelling reaction for both procedures was stopped by adding 2µl of 0.2M EDTA (pH8.0) and /or heating at 65°C for 10 minutes.

2.2.2.4.7. Hybridization protocol for Southern blots

Non-radioactive and radioactive DNA probe hybridization was carried according to the instruction manuals provided by Boehringer Mannheim and Anachem respectively. The hybridisation buffers were different for the two protocol. In non-radioactive hybridization, the buffer was made of 5XSSC, 0.1% (w/v) N-lauroylsarcosine, 0.02% (w/v) SDS, and 1% blocking solution (10% (w/v) blocking reagent; 0.1M maleic acid; 0.15M NaCl), while for radioactive hybridization, the buffer was made of 4XSSC, 0.1% (w/v) heparin (Sigma Chemicals - St Louis, USA), 0.2% (w/v) SDS, and 0.1% (w/v) tetra-sodium pyrophosphate (Sigma Chemicals - St Louis, USA).

The principle of hybridisation for both protocols was similar, therefore, the following step were carried out. The Southern blotting membranes were initially pre-hybridised in hybridization buffer within a plastic container in a shaking water bath or in the roller tubes in Hybaid oven (Hybaid Limited - Middlesex, UK) at 68°C for one hour. After that, the prehybridization solution was discarded and hybridization solution prewarmed to 68°C was added together with fully denatured DNA probe. The hybridization process continued for a total of 16 hours at 68°C.

After hybridisation membranes were washed in the following manner; 2 times each 5 minutes in ample 2XSSC, 0.1% SDS at room temperature, followed by 2 times each 15 minutes in ample 0.1XSSC, 0.1% SDS at 68°C with constant agitation. In the case of radioactive hybridization, an extra wash in 1XSSC, 0.1% SDS for 30 minutes at 68°C between the two above washes was employed.

2.2.2.4.8. Detection of hybridised fragments

2.2.2.4.8.2. Non-radioactive blots

The hybridised probes were detected immunologically with a rabbit antigen to digoxigenin conjugated to alkaline phosphatase, the bound conjugate was detected by addition of a colorimetric substrate; nitroblue tetrazolium salt in

dimethylformamide (NTB) together with X-phosphate (5-bromo-4-chloro-3-indolyl phosphate toluidinium salt). Positive hybridization was indicated by the development of purple colour within a few minutes and allowed to continue for about 16 hours in darkness. Colour development was stopped by washing the membrane in distilled water for 15 minutes.

2.2.2.4.8.2. Radioactive blots

After stringent washing the membranes were wrapped in cling film and placed in an autoradiography film cassette using Curix RP1, X-ray film (Agfa - Belgium). The film was exposed for one to two hours and developed in an automatic X-O Graph developer (X-O Graph, Image System - Malmesbury, UK). If a desirable signal was not attained another film was loaded into the cassette and exposed for a longer duration usually overnight.

Documentation of the resultant banding patterns was by photography of the blots or autoradiographic films.

2.2.2.4.9. Stripping of the probes

In order to re-use the membranes, the probes had to be removed at the end of each experiment. The methods for removing DNA probes are indicated in the respective manuals of Boehringer Mannheim and Amersham for blots hybridised with non-radioactive and radioactive probes. Briefly, the DIG labelled probes were removed by agitating the blot in a large beaker containing N,N dimethylformamide (Sigma Chemicals - St Lous, USA) at 60°C within a fume cabinet, until the purple/blue colour disappeared. The membrane was rinsed in distilled water and further washed in 0.2M NaOH, 0.1% SDS at 37°C for 2 X 20 minutes. The membrane was rinsed briefly in 2XSSC before the second pre-hybridization or sealed in a placed in plastic bag and stored at 4°C.

Regarding blots with radioactive probes, these were incubated at 45°C for 30 minutes in 0.4M NaOH, and then transferred to a solution containing 0.1XSSC, 0.1% (v/v) SDS and 0.2M Tris-HCl pH7.5 and incubated for a further 15 minutes at the same temperature. A radioactivity monitor was used to check for presence of any probe

remaining on the blots. After successful removal of the probe the membrane was rinsed with 2XSSC, sealed in a plastic bag, sealed and stored at 4°C, or subjected to the pre-hybridisation procedure if re-used immediately.

2.2.2.5. PCR fingerprinting - spoligotyping

Spoligotyping is a DNA typing method based on the DNA polymorphism found at a unique chromosomal locus, the “Direct Repeat” (DR) region found at one intergration site of IS986. The method, which involves amplification of spacer oligonucleotides between the DR and subsequent hybridization, has been described by Kamerbeek *et al.* (1994).

2.2.2.5.1. Spacer region PCR

In carrying out spoligotyping, the first step is to amplify the spacer regions found in strains by PCR. The two primers, forward primer M7921 (5' CCG AGA GGG GAC GGA AAC 3') and biotinylated reverse primer M7922 (5' biotin-GGT TTT GGG TCT GAC GAC 3') anneal to the DR and allow amplification of the spacer(s) between the DR targets (Figure 2.4). Products of different length are generated and each of the product contains a varying number of spacers and DR's depending on where primers anneal to DR's (Figure 2.4). Since the reverse primer is biotinylated, the amplified oligonucleotide strands are biotin labelled and this allows easy detection of the product by ECL detection kit (Kamerbeek *et al.*, 1994).

The PCR was carried out as indicated by Kamerbeek *et al.* (1994), except that the primer concentration was modified. The reaction mixture consisted of 75pmol of each of the primer, 0.4mM dNTPs, 0.5U *Taq DNA* polymerase, 1XPCR reaction buffer and water to a total volume of 50µl. The PCR protocol had the following steps: initial denaturation at 96°C for 3 minutes followed by 30 cycles each with denaturation at 96°C for 1 minute, annealing at 55°C for 1 minute and extension at 72°C for 30 seconds.

The PCR product was then checked by electrophoresis of 10µl of PCR product added with 2µl of loading buffer on 2% agarose gel and electrophoresed for 30 minutes. An intense smear was indicative of positive amplification.

Detection of the amplified spacer was done by hybridization of PCR product to the membrane on which oligonucleotides that are derived from known spacers are immobilized (Kindly supplied ready to use by Dr. J.D.A. van Embden).

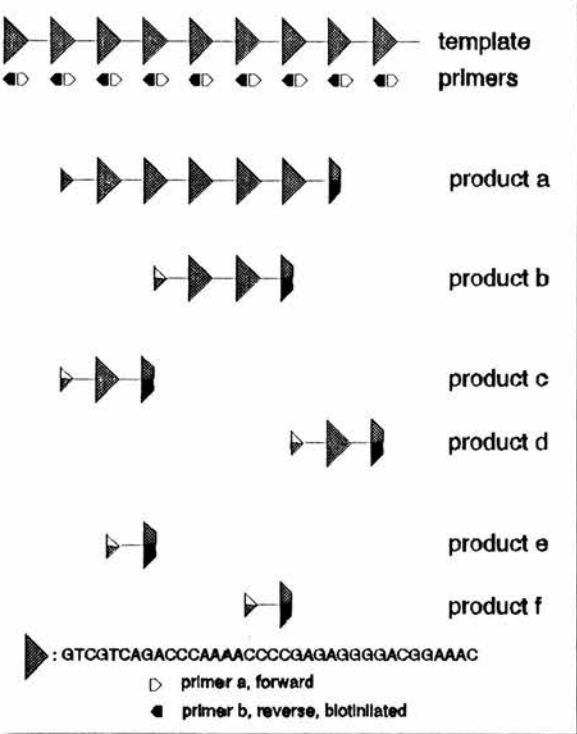


Figure 2.4. A stylised diagram showing the principle of spoligotyping

▶ = DR

2.2.2.5.2. Hybridization of PCR product

Hybridisation and detection was carried out as recommended by Kamerbeek *et al.* (1994). In short, the membrane was washed initially for 5 minutes at 55°C in 2XSSPE (diluted from 20XSSPE - 3.6M NaCl; 0.2M NaH₂PO₄; 20mM EDTA, pH7.2) supplemented with 0.1% (w/v) SDS. Then the membrane was placed into the Miniblotter® 45 (Immunetics - Cambridge, UK) in such a way that the slots are perpendicular to the line pattern of the applied unique nucleotide sequences for each spacers found in between adjacent DRs. Each perpendicular line represented a single spacer of a total of the 43 spacers. Excess liquid was removed from the slots by aspiration. 20µl of the PCR product was added to 150µl of 2XSSPE and 0.1%SDS, heat denatured by boiling for 10 minutes and then quenched on ice. Prior to loading into the miniblotter, the mixture was thoroughly mixed by vortexing and 140µl loaded. The open ends of the slots were sealed using masking tape to avoid slot-to-slot contamination and hybridization was performed at 60°C for one hour. After hybridisation, the slots were emptied by aspiration, followed by brief washing of the used slots by filling them with 2XSSPE and 0.1% SDS. The miniblotter was dismantled and the membrane removed and washed by gently agitating in a tray containing 250ml of 2XSSPE, supplemented with 0.5% SDS pre-warmed to 60°C for 10 minute at 60°C.

2.2.2.5.3. Detection of hybrids

The hybridised PCR product was detected with peroxidase labelled streptavidin that binds the biotin which is present in the PCR product. For chemiluminiscent detection of hybridizing DNA, the ECL detection kit (Amersham International plc. - UK) was used. The peroxidase present on streptavidin catalyses a reaction that results in the emission of light and light is detected by exposure of the membrane to an X-ray film. Step by step procedures for detection were carried out as follows. The membrane was incubated at 42°C for 45 minutes in a solution of 2.5µl streptavidin-peroxidase conjugate (Boehringer Mannheim - Germany) in 10ml of 2XSSPE, supplemented with 0.5% SDS,. The membrane was then washed by agitating for 10 minutes at 42°C

in 150ml of 2XSSPE, supplemented with 0.5% SDS. The membrane was washed briefly in 2XSSPE solution before incubating in 20ml ECL detection liquid (1:1 ECL solution 1 and 2) for exactly one minute. The membrane was and placed in a plastic bag before inserting in a X-ray film cassette with a Hyperfilm-ECL film (Amersham International plc. - UK) for 1 minute. The exposed film was replaced by another film in case a longer exposure is required. The exposed film was then developed as described above and documented by photography.

2.2.2.5.3. Stripping of membranes

The membranes were incubated in roller tubes containing 1% (w/v) SDS at 80°C for one hour, then washed in 20mM EDTA pH8.0 for 15 minutes at room temperature. The membranes were then sealed in plastic bag, sealed and stored at 4°C.

2.2.2.6. Cloning of RAPD PCR fragments

Further analysis of RAPD products (Chapter 5) was performed by cloning and sequencing.

DNA fragments were excised from 0.8% LMP agarose and the DNA recovered as described in 2.2.2.4.4. The quantification of DNA also was determined as described in 2.2.2.2. The fragments were cloned using the pGEM[®]-T vector SystemII (Promega - Madison, USA) which is suitable for cloning PCR products because the 3'-T overhangs at the insertion site complement produced by PCR at the 3' ends of the DNA fragments (Promega Technical Bulletin 10/94).

The DNA fragments were ligated into pGEM[®]-T after, the amount of DNA fragment (insert) to be ligated according to the following formula (Promega Technical Bulletin 10/94):

$$[\text{ng of insert} = \{(\text{ng of vector} \times \text{kb size of insert}) \times (\text{kb size of vector})^{-1}\} \times \text{insert:vector molar ratio}]$$

Two insert:vector molar ratios were used in this experiment *viz.* 1:1 and 5:1 so as to maximize the chances of ligation. The reaction was carried out at 16°C for 3 hours.

E. coli JM109 High Efficiency Competent Cells (Promega - Madison, USA) were used for transformation.

The protocol for transformation was carried out in accordance with manufacturer's manual (Promega Technical Bulletin 10/94). Briefly, five and 10µl of the ligation reaction for respective 1:1 and 5:1 insert:vector molar ratio were aliquoted into a sterile 1.5ml eppendorf placed on ice and 50µl of JM109 competent cells were added carefully. The tubes were gently flicked to mix the contents and returned to ice for a 20 minutes incubation. In order to enhance transformation, the cells subsequently were heat shocked by placing the tube for 45 seconds in water bath set exactly at 42°C and then returned to ice for a further 2 minutes incubation. 1400µl of Luria-Bertani (L-B) broth (1% (w/v) Bacto-tryptone; 0.5% (w/v) Bacto-yeast extract; 0.5% (w/v) NaCl, supplemented with 0.1% (v/v) ampicillin, 0.08% (w/v) X-gal and 0.12% (w/v) IPTG) were added to each eppendorf, gently mixed and incubated for one hour at 37°C. Two aliquots of transformed *E. coli* (50 and 150µl) from each eppendorf were spread on two duplicate sets of L-B agar (L-B broth added with 0.15% (w/v) agar) plates and incubated at 37°C overnight.

2.2.2.7. Sequencing of cloned *f2b* and *f3b* DNA fragments

The sequencing of the two fragments (Chapter 5) was carried out by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the Sequenase® Version 2.0 DNA sequencing kit (United States Biochemical - Ohio, USA).

4µg plasmid DNA, recovered as described above was denatured by the addition of NaOH to a final concentration of 0.4M and incubated at 37°C for 30 minutes. The denatured DNA was precipitated by adding 1/10 volume of 3M NaOAc and 2 volumes of chilled ethanol, and placed at -20°C overnight. Precipitated DNA was pelleted by centrifugation at 13,000g for 10 minutes. The supernatant was discarded and the DNA pellet air dried.

Sequencing reactions were performed as indicated in the instruction manual (United States Biochemical). Compression is a common feature in sequencing of mycobacterial DNA, which is G-C rich (62 -65%) (Dr. K. Stevenson personal

communication 1996) but this problem was overcome by the use of high enzyme concentration and electrophoresis on formamide gels. New primers were designed and used to override any remaining compressions.

2.2.2.8. Sequencing gel electrophoresis

As recommended by the manufacturer (United States Biochemical - USA) A 1X Glycerol Tolerant Gel Buffer (GTGB) (89 mM Trizma base; 28.5 mM Taurine; 0.5 mM EDTA) was used for making gels and for electrophoresis. This buffer allowed the use of reaction containing high concentrations of glycerol which makes the enzyme more stable (Dr. K. Stevenson - personal communication). For the purpose of this experiment a 6% gel was used.

Electrophoresis was performed using a Model S2 Electrophoresis apparatus and power supply (GIBCO-BRL - Gaithersburgh, MD, USA). Prior to running the samples, the gel was pre-run for 10 minutes at 150W and a constant current of 40mA, using 1XGTGB as electrophoresis buffer. The sequencing reactions were divided into two portions to allow a longer run of approximately 4 hours and a shorter run of 3 hours. Fully denatured sequencing reaction mixtures from individual nucleotide termination mixes were loaded into adjacent lanes marked on the glass plate (*e.g.* AGCT).

After electrophoresis, the gel was fixed in 5% glacial acetic acid and 20% methanol for 15 minutes and dried onto Whatman 3MM filter paper using a Slab Gel Drier SE1160 (Hoefer Scientific Instruments - San Francisco, USA) at 80°C for 1 to 1½ hours.

After drying, the gel was placed in an X-Ray film cassette and in the dark, a Curix, RP1 Agfa autoradiography film (Agfa - Belgium) exposed overnight. The exposed film was processed as described above.

The sequences were read manually on the light box, and the sequences recorded.

CHAPTER 3
EPIDEMIOLOGY OF BOVINE TUBERCULOSIS
IN MAN AND CATTLE IN TANZANIA

CHAPTER 3. EPIDEMIOLOGY OF BOVINE TUBERCULOSIS IN MAN AND CATTLE IN TANZANIA

3.1. INTRODUCTION

3.1.1. BOVINE TUBERCULOSIS IN CATTLE

The history of bovine tuberculosis in both cattle and man in Tanzania, though scant, has been extensively reviewed in Chapter 1. It appears that the disease has been prevalent in a number of foci in different locations within the country. The Southern Highlands, particularly the Usangu Plains, have continuously been recording a high proportion of cattle with tuberculous lesions at slaughter. In a nation-wide survey (Kazwala *et al.*, 1993) it was found that the proportion of lesion-positive cattle in regions outwith the Usangu Plains was between 0.1 to 2%, whereas in regions encompassing the Usangu Plains (Mbeya and Iringa), the regional average was between 4 and 10% (Kazwala *et al.*, 1993). Studies involving tuberculin testing of cattle were mainly confined to government farms keeping grade or exotic breeds whose population is approximately 2% of the national herd. In most of those studies the prevalence of the disease was below 1.0%. A high reactor rate (12%) was recorded in dairy farms belonging to missionaries in Mtwara and Lindi regions in the Southern Zone of Tanzania. These herds have been known to be infected for many years (Markham 1952) and since the owners have been reluctant to cull infected animals the incidence of disease in those farms has increased year after year.

3.1.2. ZOONOTIC IMPORTANCE OF *M. BOVIS*

The zoonotic importance of *M. bovis* in Tanzania, has yet to be elucidated fully. Early reports by Cornell (1934), Markham (1952) and Wilson (1954) had raised concerns of transmission of tuberculosis from cattle to man. These workers noticed cultural habits such as living with cattle in sub-terrain houses and drinking of raw milk by people in various part of the country as contributory factors. They, therefore,

indicated that there was a likelihood of transmission of not only tuberculosis, but other zoonoses, between cattle keepers and their animals. Studies to ascertain the importance of tuberculosis in the human population in Tanzania were those carried out by the East African and British Medical Research Council (EA/BMRC) in 1969/70 and by Tanzania/British Medical Research Council Collaborative Study (T/BMRC) in early 1980's (EA/BMRC 1975; T/BMRC 1985). In those investigations, attempts were made to determine the proportion of cases of pulmonary and/or of extra-pulmonary tuberculosis. The two studies found that between 7.9 to 12.6% of tuberculosis cases encountered were extra-pulmonary. The EA/BMRC study found mycobacteria in 59.6% of the 3690 sputum specimens but no effort made to identify which species of mycobacteria were involved. The T/BMRC study found that 68.3% of 1006 sputums were positive for *M. tuberculosis*. The National Tuberculosis and Leprosy Programme (NTLP) was initiated in Tanzania in 1977, the main purpose of which was the prevention of these diseases in the human population by early detection and subsequent chemoprophylactic intervention. With this programme all new cases of tuberculosis encountered in all districts of Tanzania were recorded in NTLP registers. These registers were scrutinised by Kazwala *et al.* (1993). It was found that 16% of all cases of tuberculosis in Tanzania were the extra-pulmonary form, and that a high proportion of these cases occurred in regions which had a high cattle to human ratio (Catley 1992). Recently Richter *et al.* (1991) investigated the involvement of mycobacteria in cases of extra-pulmonary tuberculosis diagnosed at Muhimbili Medical Centre, in Dar es Salaam. They confirmed 18% of the 271 cases to be due to mycobacteria by combined bacteriology and histology, but did not type any strains.

3.1.3. AIMS OF THE STUDY

The aims of the current study were (i) to determine the presence of *M. bovis* in cases of bovine tuberculosis in cattle after tuberculin test and post-mortem examination, (ii) to determine the presence *M. bovis* infection in man by culture, and (iii) to ascertain the mode of transmission between cattle and man in pastoralist communities

3.2. RESULTS

Materials and methods pertinent to this chapter have been described in Chapter 2.

3.2.1. BOVINE TUBERCULOSIS IN CATTLE

3.2.1.1. Tuberculin testing

3.2.1.1.1. Validation of the tuberculin test.

In order to validate the performance of the tuberculin test as carried out in the Usangu Plains, 35 tuberculous tissues from tuberculin tested cattle were selected at random and sent to SMRL for cultivation of mycobacteria. The results of the culture and tuberculin reactivity are summarised in Table 3.1. The test had a sensitivity of 75%, positive predictive value of 66.7% and a negative predictive value 92.3%. As regards specificity testing; a tuberculosis free herd of 150 cattle belonging to the Sokoine University of Agriculture was tested in a similar manner to cattle tested in the Usangu Plains and no reactor was found. This finding indicated that the SICTT as applied in Tanzania was 100% specific.

Table 3.1.. Validation of performance of tuberculin test in the Usangu Plains.

Tuberculin test	Isolation of <i>M. tuberculosis</i> complex*		
	positive	negative	total
positive	6	3	9
negative	2	24	26
total	8	27	35

*Isolates were confirmed to be members of *M. tuberculosis* complex; further identification was not carried out.

3.2.1.1.2. Tuberculin testing of cattle in the Usangu Plains

A total of 911 out of 6383 (14.3%) cattle tuberculin tested in the Usangu plains, were identified as reactors using a severe interpretation of results (i.e. bovine bias >2mm). 1690 cattle (28.5%) gave an inconclusive reaction, whilst 3465 (54.3%) cattle were negative to the test. A distribution of reactors according to their geographical areas

(Table 3.2.) shows that stations in the middle section of the plains, the Mbarali sub-district of Mbeya region (i.e. Rujewa, Chimala, Igurusi and Middle Usangu areas) had >10% cattle classified as reactors (13.6-18.7%). Stations on the periphery of the plains i.e. Uyole in Mbeya district, Makambako and Njombe in Njombe district had lower reactor rates (<10.0%).

Table 3.2. Distribution of reactor cattle according to geographical location

Station	Total tested	reactors (%)	Inconclusive (%)
Igurusi	774	145 (18.7)	252 (32.6)
Mid-Usangu	2559	421 (16.5)	547 (27.7)
Chimala	865	121 (14.0)	224 (25.9)
Rujewa	1068	145 (13.6)	276 (25.8)
Uyole	269	27 (10.0)	71 (26.4)
Makambako	457	40 (8.7)	138 (30.2)
Njombe	391	12 (3.1)	123 (31.5)
Total tested	6383	911 (14.3)	1690 (28.5)

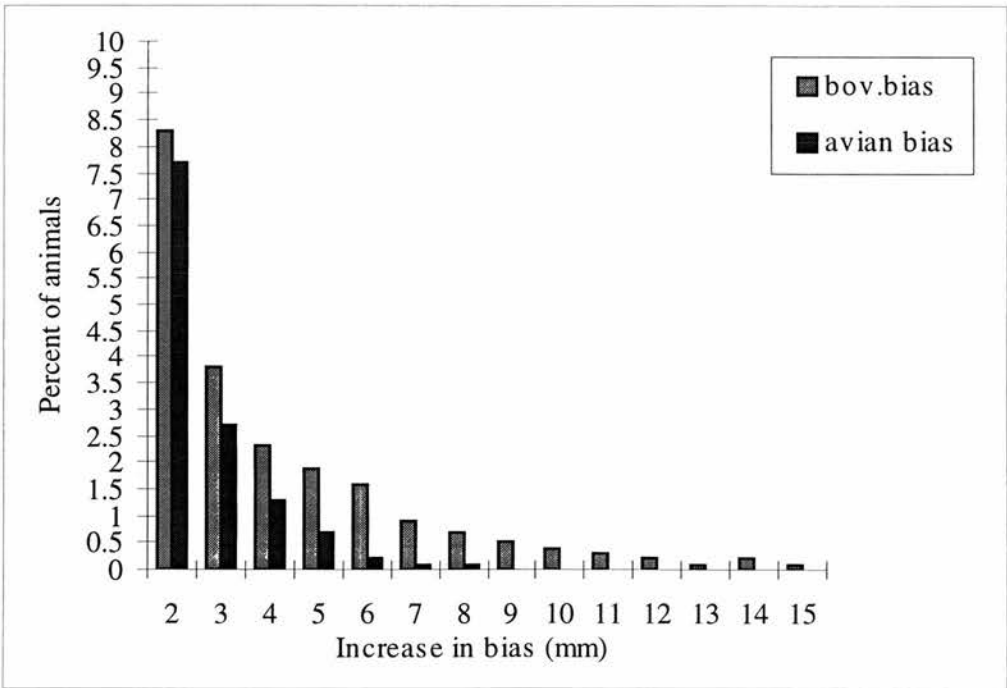


Figure 3.1. The relative reactivity of cattle to bovine and avian PPDs

The comparison of the level of reactivity to bovine and avian PPDs (Figure 3.1). indicates that there were more cattle reacting to bovine PPD with a bias of up to 15mm, this finding shows the importance of infection due to *M. bovis* in the Usangu Plains. However, there were another 329 (5.1%) cattle which reacted more to avian PPD than bovine PPD. This observation implies that cattle in this region are also affected with either atypical mycobacteria species or other micro-organisms of related genera such as *Norcadia*, *Corynebacterium*, and *Rhodococcus*. These micro-organisms are likely to interfere with the performance of the tuberculin test by rendering infected animals classified as inconclusive reactors. This observation is manifested by the percentages presented in Table 3.2., where the number of cattle with inconclusive reaction (28.5%) was twice the number of cattle classified as reactors (14.3%).

A total of 239 herds of cattle encountered in the course of this study had a mean herd size per station ranging from 8 to 54 cattle. The distribution of reactors amongst the herds examined at different parts of the Usangu plains show that 122 (51%) herds had reactor cattle (Table 3.3). Herds examined in the stations of Mbarali sub-district were more affected (53.6 - 93.3%) than those of other districts (4.3 - 30.3%). This difference was found to be statistically significant ($p < 0.001$; $\chi^2 = 46$; $df = 1$; $p = 0.0001$). The trend of the disease as assessed on a herd basis shows a strong positive correlation ($r = 0.85$) between the herd size and percentage of herds positive (Figure 3.2), while a similar but moderate positive correlation ($r = 0.58$) was depicted when herd size was compared to number of cattle infected (Figure 3.2).

A large proportion of cattle examined in the course of the current study were those of the Mid-Usangu areas (2559 of 6383 $\cong 40\%$). These cattle were from five villages (Table 3.4) situated at least 50km apart. 42 of 45 herds belonging to these villages had reactor cattle and the mean percentage reactor cattle per village was found to be 16.5% (range 10 - 28.5%). The difference between the number of cattle found to be reactors per village was statistically significant ($p < 0.001$; $\chi^2 = 108.7$; $df = 4$; $p = 0.0001$).

Table 3.3. Distribution of reactor herds according to geographical location

Station	Total herds tested	Mean herd size (range)	Herds positive (%)
Mid-Usangu	45	54 (6 - 162)	42 (93.3)
Igurusi	12	40 (3 - 184)	9 (75.0)
Rujewa	35	14 (2 - 75)	19 (54.5)
Chimala	56	15 (2 - 84)	30 (53.6)
Makambako	56	8 (2 - 23)	17 (30.3)
Njombe	12	21 (6 - 58)	3 (25.0)
Uyole	23	8 (2 - 22)	1 (4.3)
Total	239		122 (51.0)

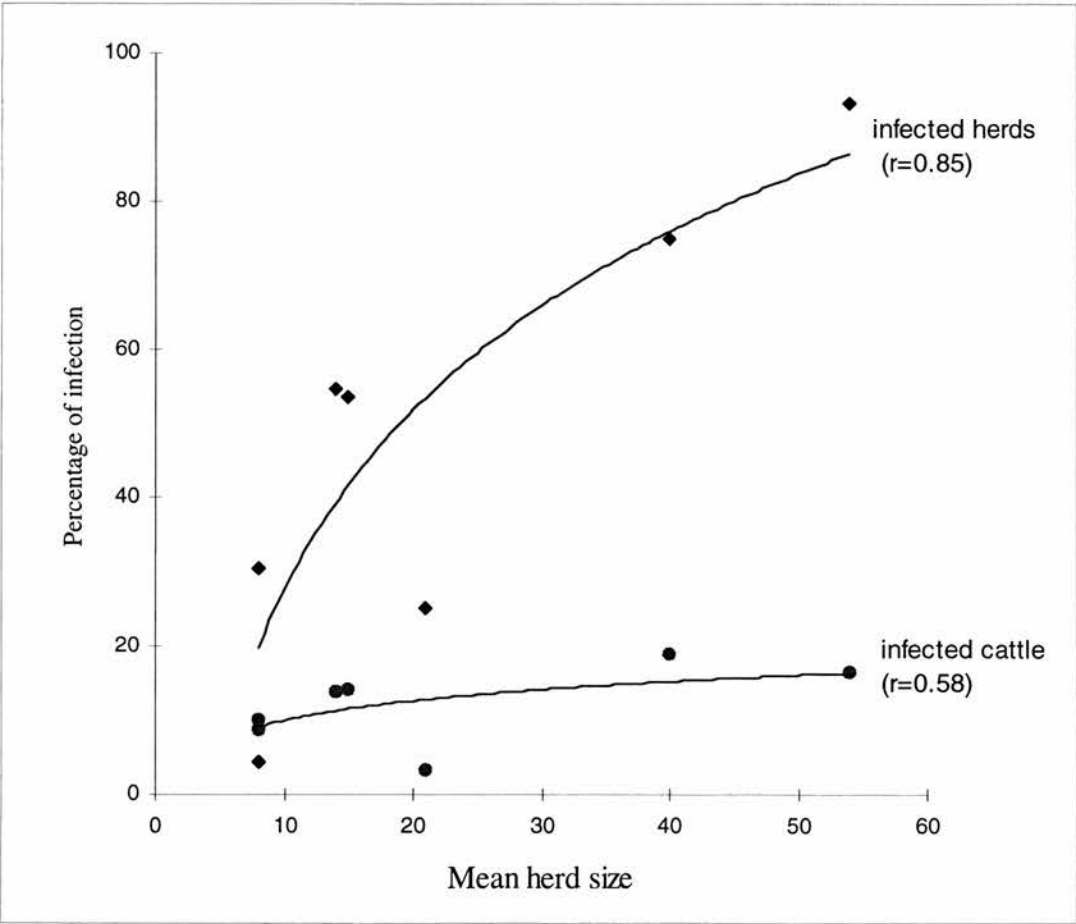


Figure 3.2. The effect of herd size on the incidence of bovine tuberculosis in herds and cattle.

Table 3.4. The distribution of reactors cattle tested in the Mid-Usangu area by village of origin.

Village	Herds tested	animals tested	mean herd size (range)	positive herds (%)	positive cattle (%)
Matebete	12	652	54(14-103)	12 (100)	186(28.5)
Muongano	3	212	71(37-98)	3 (100)	40 (18.9)
Mawindi	3	238	79(48-141)	3 (100)	42 (17.6)
Upagama	4	311	78(24-162)	3 (75.0)	38 (12.2)
Mwanavala	23	1146	45(6-143)	21(91.3)	115(10.0)
Total	45	2559	54(6-162)	42 (93.3)	421 (16.5)

Matebete village, a focus of study of zoonotic importance in man, had all 12 herds positive and number of cattle found to be reactors was twice the figure for the entire study area (14.3%; Table 3.2).

In order to study the zoonotic importance of bovine tuberculosis in a population of pastoralists, 12 herds of cattle belonging to families willing to participate in this study were examined. Tuberculin reactivity of these twelve cattle herds is shown in detail in Table 3.5. All twelve herds were positive, the mean percentage reactor rate per herd was 30.4% (range 8.8 - 53.6%).

Despite the fact that only two families had no history of tuberculosis, cattle belonging to these families had significantly lower reactor rates (8.8 -20%) compared to those belonging to families with a history of tuberculosis amongst its members (20.2 - 53.3%) ($p<0.001$; ($\chi^2=13.22$; $df=1$; $p=0.001$)).

Table 3.5. Results of tuberculin testing of cattle at Matebete Village

Family ID	History of human tuberculosis	Total cattle tested	Reactors (%)
RK	present	28	15 (53.6)
JKS	present	17	8 (47.1)
*YM	present	103	43 (41.7)
KKN	present	34	14 (41.2)
SKK	present	59	20 (33.9)
DEK	present	59	19 (32.2)
*PS	present	44	10 (22.7)
SK	present	88	19 (21.6)
KK	present	14	3 (21.4)
MGK	present	99	20 (20.2)
PK	absent	50	10 (20.0)
SKN	absent	57	5 (8.8)
Total		652	186 (28.5)

**M. tuberculosis* isolated (see 3.2.2.1)

3.2.1.1.3. Assessment of tuberculin test performance in different categories of cattle kept at the Usangu Plains.

In the course of carrying out this study, a number of variables were taken from individual animals as indicated in Table 3.6. These variables were taken in order to assess their effect on the performance of the tuberculin test. The first variable to be assessed was the effect of age of the cattle. Tuberculin tested cattle were categorised into three main groups as shown in Table 3.6. The reactor rate amongst the groups differed very significantly ($p < 0.001$) with older cattle being more affected than younger cattle and calves (15.2% vs. 9.7 and 9.1%).

Regarding breeds of cattle, in the Usangu Plains there are several government institutions which keep exotic breeds (i.e. Friesian, Ayrshire, Guensey, Jersey etc.) of cattle for milk production or as breeding units for producing crosses of these breeds with indigenous breeds (Short Horn Zebu - SHZ). Some of these animals were

Table 3.6. Comparison of the effect of various cattle variables on tuberculin reactivity of cattle in the Usangu Plains.

Variable	Number of reactors (%)	Level of significance (Chi-square statistics)
1. Age		$p < 0.0001^{***}$ ($\chi^2 = 41.53$; $df = 2$; $p = 0.00001$)
Calves <18 months (n=1211)	110 (9.1)	
Cattle 1½-3yrs (n=855)	83 (9.7)	
Cattle >3yrs (n=3723)	570 (15.2)	
2. Sex		$p < 0.05^*$ ($\chi^2 = 6.02$; $df = 1$; $p = 0.014$)
Female (n=4039)	509 (12.6)	
Male (n=1814)	268 (14.8)	
3. Breed		$p < 0.05^*$ ($\chi^2 = 5.48$; $df = 1$; $p = 0.0192$)
Exotic breeds (n=244)	20 (8.2)	
Short Horn Zebu (n=5692)	761 (13.4)	
4. Male cattle		$p < 0.01^{**}$ ($\chi^2 = 8.65$; $df = 1$; $p = 0.0033$)
Entire bulls (n=922)	114 (12.4)	
Castrated (oxen) (n=892)	154 (17.3)	
5. Pregnancy		$p > 0.05^{NS}$ ($\chi^2 = 0.11$; $df = 1$; $p = 0.7369$)
Pregnant (n=493)	59 (12.0)	
Non-pregnant (n=3600)	450 (12.5)	
6. Lactation		$p < 0.05^*$ ($\chi^2 = 5.58$; $df = 1$; $p = 0.0181$)
Lactating (n= 1627)	237 (14.6)	
Non-lactating (n=2079)	248 (12.0)	

*** = highly significant difference ** = moderately significant difference

* = slightly significant difference ^{NS} = No significant difference

tuberculin tested in a similar manner to SHZ tested in the same locality. The reactor rate among the indigenous cattle (13.4%) was significantly higher ($p < 0.05$) than exotic breeds of cattle (8.2%).

There was a sex variation among the reactor cattle, male cattle were significantly ($p<0.05$) more affected by tuberculosis than the female cattle (14.8% vs. 12.6%). Amongst the male cattle, there was a significant difference in reactivity of castrated cattle and bulls to the tuberculin test. Castrated cattle, which are more often kept for draught power by livestock keepers, were significantly ($p<0.01$) more affected by tuberculosis than bulls (17.3% vs. 12.4%). Among the female cattle, the effect of the pregnancy and lactation to reactivity to the tuberculin test was assessed. While pregnancy appeared not to alter the reaction to the tuberculin test, lactation was found to significantly ($p<0.05$) affect the reactivity (Table 3.6).

3.2.1.2. Post-mortem examinations

Tuberculous lesions were found in 67 of 841(8.0%) cattle at routine meat inspection, while at an enhanced post-mortem procedure (i.e. slicing tissues at 2mm interval), a total of 225 (26.8%) cattle were declared to have tuberculous lesions (Table 3.7). A total of 199 carcasses inspected in the course of this study were found to have granulomatous lesions on the external surfaces of the thoracic segment of the aorta. 63 (31.7%) of these carcasses had visible lesions in other organs as well.

Table 3.7. The distribution of cattle with visible lesions (VL) at different stations

Station	Total cattle slaughtered	VL* at routine meat inspection (%)	VL* at enhance meat inspection (%)
Chimala	86	5 (5.8)	32 (37.2)
Igurusi	151	9 (6.0)	40 (26.5)
Makambako	52	8 (15.4)	10 (19.2)
Njombe	205	10 (4.9)	40 (19.5)
Rujewa	226	29 (13.8)	58 (25.6)
Uyole	121	6 (5.0)	45 (37.2)
Total	841	67 (8.0)	225 (26.8)

*adjusted by removal of animals with aortic lesions only.

The distribution of tuberculous lesions in the carcasses examined is summarised in Table 3.8. The lesions were present in many sites but the most commonly affected were the lymph nodes associated with the respiratory tract (16.9-36.8%), the head (20-28.9%) and the alimentary tract (15.5%). Generalised tuberculosis was encountered in only two cases (0.9%). This analysis excludes lesions found in the aorta.

Table 3.8. Distribution of tuberculous lesions in lesion positive cattle (n=225)

Organ/tissue	Number with VL	Proportion to total VL (%)
Retropharyngeal ln	65	28.9
Sub-mandibular ln	45	20.0
Cranial mediastinal ln	61	27.1
Caudal mediastinal ln	94	36.8
Left bronchial ln	72	32.0
Right bronchial ln	38	16.9
Mesenteric ln	35	15.5
Other carcass lnn	3	1.3
Lungs	27	12.0
Liver	3	0.9
Spleen	1	0.4
Kidney	1	0.4
Generalised tb	2	0.9

ln = lymph node lnn = lymph nodes

3.2.1.3. Comparison of post-mortem and tuberculin test results

Although all 841 cattle slaughtered at the stations mentioned above were injected avian and bovine PPDs on day 0 of tuberculin test, only 780 (92.7%) cattle completed the test. The remaining 61 (7.3%) cattle were slaughtered before 72 hour reading of the tuberculin reaction. 80 (10.2%) of 780 cattle were classified as reactors, and there was a highly significant difference in the presence of visible

lesions detected at routine and enhanced meat inspection ($p<0.0001$; ($\chi^2=51.58$; $df=1$; $p=0.00001$)). Seventeen (21.3%) compared to 56 (70.0%) of the 80 reactor cattle were found to have visible lesions at routine and enhanced meat inspection respectively (Table 3.9).

Table 3.9. The ability of tuberculin test to detect lesion positive at routine and enhanced post-mortem

Tuberculin test	Routine meat inspection		Total
	Visible lesion	No visible lesion	
Positive	17	63	80
Negative	50	650	700
Total	67	713	780

Tuberculin test	Enhanced meat inspection		Total
	Visible lesion	No visible lesion	
Positive	56	24	80
Negative	168	532	700
Total	224	556	780

Comparison of tuberculin reactivity of cattle with tuberculous lesions to avian and bovine PPDs individually is summarised in Figure 3.3. Among the 224 cattle with tuberculous lesions, 119 (53.1%) and 95 (42.4%) did not react to avian and bovine tuberculin respectively. The proportion of cattle with visible lesions which reacted to bovine PPD, showed increase in skin thickness of up to and above 12mm, compared to increase in skin thickness due to reaction caused by avian PPD which was up to 7mm (Figure 3.3).

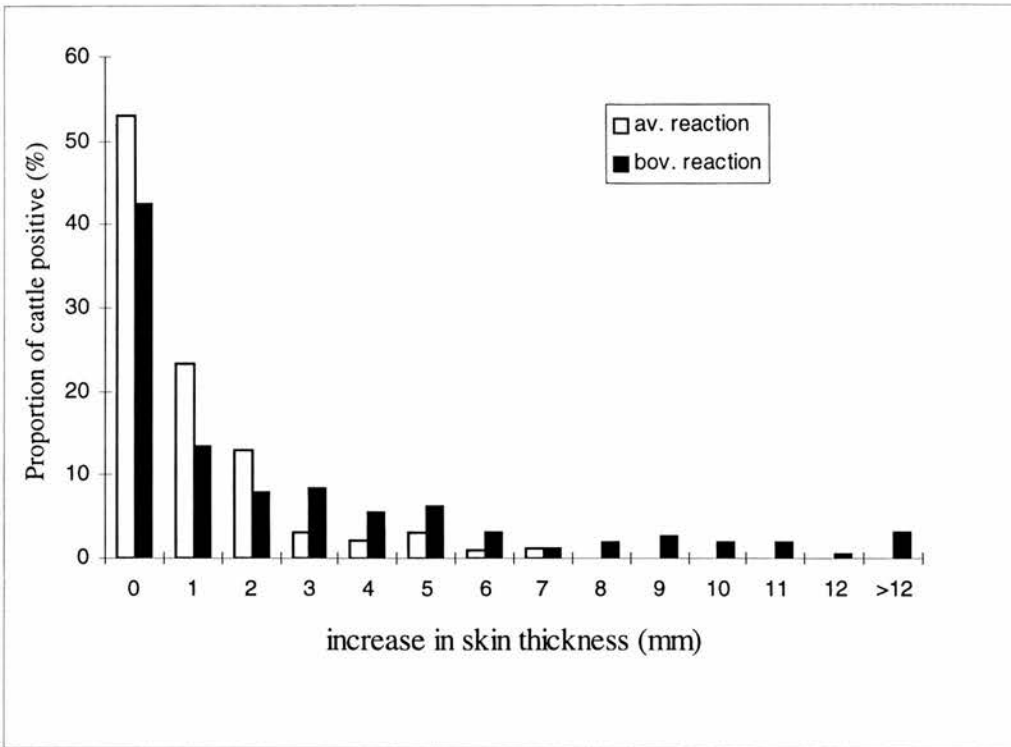


Figure 3.3. The reactivity of 224 lesion positive cattle with completed tuberculin test

3.2.1.4. Bacteriology of tissues from cattle

A total of 1068 samples were submitted for culture from cattle slaughtered during the tuberculin test validation trial in Arusha. Samples gathered from the Usangu Plains included; 883 milk samples (26 from Matebete village) and 849 pools of lymph nodes. Results of isolation of mycobacteria as performed at SUA laboratory are presented in Table 3.10. It should be noted that a total of 198 lymph node specimens from the Usangu Plains was discarded after being found putrefied in the course of storage at field stations and SUA laboratory. Also 78 milk samples were not processed after their containers were found to have leaked.

According to the isolation and preliminary identification as performed by the SUA laboratory, 4.3% of 1068 tissues from cattle slaughtered at the Arusha abattoir were positive for mycobacteria. All except one of the 46 isolates were tentatively classified as members of the *M. tuberculosis* complex. As regards specimens from the Usangu plains, a total of 58 strains were cultured from various specimens as shown in Table 3.10. Only 10 (17.2%) strains were provisionally identified as members of the *M.*

tuberculosis complex, the rest were classified as MOTTs. Five of 26 (19.2%) samples of milk from cattle belonging to the Matebete village pastoralists, yielded mycobacteria, and all of them were identified as MOTTs by SUA laboratory.

Table 3.10. Isolation and preliminary identification of mycobacteria from cattle specimens by SUA laboratory.

Study area	Specimen	Total processed	total isolates (%)	MTBC	MOTTs
Arusha	lymph nodes	1068	46 (4.3)	45	1
Usangu Plains	milk	779	31 (3.9)	2	29
	lymph nodes	651	27 (4.1)	8	19
Matebete	milk	26	5 (19.2)	0	5

MTBC = Mycobacterium tuberculosis complex MOTTs = Non-tuberculous mycobacteria

As part of quality control measures, all strains cultured at the SUA laboratory were submitted to SMRL for confirmation of diagnosis prior to submission to MRI laboratory for molecular analysis. Similarly, in addition 35 tissues from the slaughtered cattle were sent to SMRL laboratory for primary cultivation. These specimens were those intended for validation of the tuberculin test employed in the study at the Usangu Plains (Table 3.1). The identity of the strains successfully identified at SMRL is summarised in Table 3.11. There was some disagreement in the identity of the strains of mycobacteria from tissues from Arusha (Table 3.10 vs. Table 3.11), this was attributed to the inability of the SUA laboratory to fully speciate strains of mycobacteria at the beginning of the BTB project.

Table 3.11. Identification of species of mycobacteria from cattle specimens by SMRL

Mycobacterium spp.	Usangu Plains		Arusha
	Milk samples (%)	Lymph nodes (%)	Lymph nodes (%)
<i>M. bovis</i>	2 (6.5)	10 (27.0)	28 (80.0)
<i>M. tuberculosis</i>		1 (2.7)	
<i>M. avium</i>		5 (13.5)	5 (13.5)
<i>M. terrae</i>	7 (22.6)	8 (21.6)	
<i>M. flavescens</i>	13 (41.9)	1 (2.7)	
<i>M. smegmatis</i>	4 (12.9)	2 (5.4)	
<i>M. fortuitum</i>	2 (6.5)	1 (2.7)	
<i>M. kansasii</i>		2 (5.4)	1 (2.8)
<i>M. gordonae</i>	1 (3.2)		
Myco. spp.**	2 (6.5)	7 (18.9)	1 (2.8)
Total	31	37	35

** Strains from heavily contaminated cultures that could not warrant further identification

Furthermore, it should be noted that a number of strains submitted to SMRL were found to be heavily contaminated. Eleven strains from Arusha and thirty strains from the Usangu Plains could neither survive the extended decontamination procedures at both SUA and SMRL nor be subcultured at SMRL. From the 35 tissues submitted and processed at SMRL, 9 strains were identified as members of the tuberculosis complex.

3.2.2. MYCOBACTERIUM TUBERCULOSIS COMPLEX IN HUMAN

3.2.2.1. Isolation of mycobacteria

A total of 44 out of 149 (29.5%) specimens collected from human cases of tuberculosis were found to be positive for mycobacteria. There was a significant

difference ($p<0.05$; ($\chi^2=4.03$; $df=1$; $p=0.045$)) between the isolation rates of mycobacteria from pulmonary and extra-pulmonary tuberculosis. Twenty one isolates (39.6%) were cultured from 53 cases of extra-pulmonary tuberculosis, while 23 isolates (24.0%) were recovered from 96 cases of pulmonary tuberculosis. The isolation rate of mycobacteria from extra-pulmonary cases was relatively higher in samples from Arusha (80.0%) followed by samples from the Usangu Plains (38.5%) and the lowest isolation rate was from samples collected in Dar-es Salaam and Morogoro (Table 3.12).

Table 3.12. The isolation rate of mycobacteria from human cases of tuberculosis in Tanzania

Region	Form of tuberculosis				Overall isolation rate(%)
	Extrapulmonary		Pulmonary		
	total	pos. (%)	total	pos. (%)	
Arusha	15	12 (80.0)	NA	NA	12/15 (80.0)
Dar/Morogoro	25	4 (16.0)	NA	NA	4/25 (16.0)
Usangu Plains	13	5 (38.5)	96	23 (24.0)	28/109 (25.7)
Total	53	21 (39.6)	96	23 (24.0)	44/149 (29.5)

NA = No sample available.

Overall, 31 (70.5%) of 44 isolates of mycobacteria recovered from specimens from all forms of tuberculosis were classified as *M. tuberculosis*, while 7 (16.0%) isolates were identified as *M. bovis* and 6 (13.5%) isolates were classified as members of MOTTs (Table 3.13). There was no significant difference ($p>0.05$; ($\chi^2=2.81$; $df=1$; $p=0.09$)) in the isolation rate of *M. tuberculosis* from either pulmonary or extra-pulmonary cases of tuberculosis (69.1% vs. 71.4%). There was significantly high ($p<0.05$; ($\chi^2=6.03$; $df=1$; $p=0.014$)) isolation rate of *M. bovis* among strains recovered from cases of extra-pulmonary tuberculosis (28.6%) than pulmonary tuberculosis (4.3%) (Table 3.13).

Table 3.13. The distribution of *Mycobacterium* species amongst the positive cases of tuberculosis.

Mycobacterium spp.	Total isolates (%)	Form of tuberculosis	
		Extra-pulmonary(%)	Pulmonary(%)
<i>M. tuberculosis</i>	31 (70.5)	15 (71.4)	16 (69.1)
<i>M. bovis</i>	7 (15.9)	6 (28.6)	1 (4.3)
<i>M. avium</i>	1 (2.3)	0	1 (4.3)
<i>M. fortuitum</i>	1 (2.3)	0	1 (4.3)
<i>M. terrae</i>	4 (9.1)	0	4 (17.4)
Total isolates	44	21	23

* Proportion of the total isolates

A total of 20 cases examined during this study were residents of Matebete Village. There were only 4 isolations of *Mycobacterium* species. Of these, two were strains of *M. tuberculosis*, 2 were *M. terrae*, and no *M. bovis* was isolated from this village.

3.2.2.2. Assessment of risk factors involved in the spread of mycobacteria infection

In the course of this study, epidemiological information regarding each case was collected in order to assess risk factors associated with the spread of tuberculosis in man (Appendix II). In view of the fact that not all cases had their accompanying data collected, the analysis of the effect of the parameters was restricted to those cases where data regarding such parameter was available.

The correlation of the parameters and the recovery of mycobacteria is summarized in Table 3.14. Although the number of *M. bovis* isolates was small, the questionnaire was able to extract detailed information regarding risk factors associated with infection in man (Table 3.14).

As regards the difference in the isolation of mycobacteria according to the age groups, there was a significantly ($p<0.05$; $\chi^2=6.56$; $df=2$; $p=0.0377$) higher isolation rate of mycobacteria from older patients (71.4%) as compared to the other age groups (25.5

to 28.5%). However, children were found to be more affected by *M. bovis* than the older members of the population (Table 3.14).

The comparison of mycobacteria isolated from male and female is summarised in Table 3.14. The difference in the isolation rate of all mycobacteria and in particular *M. bovis* as shown in Table 3.14, was found to be insignificant ($p>0.05$; $\chi^2=1.22$; $df=1$; $p=0.269$).

There was no significant difference ($p>0.05$; $\chi^2=0.09$; $df=1$; $p=0.769$) in the percentage of mycobacteria isolated from cases vaccinated and those not vaccinated with *M. bovis* BCG (28.3% vs. 25.6%). Furthermore, there were four *M. bovis* isolates from patients who had BCG vaccination. Data on this parameter from the remaining *M. bovis* strains was not available.

Comparison was made on the prevalence of mycobacteria infection among people residing in the rural and urban areas. There was no significant difference ($p>0.05$; $\chi^2=0.34$; $df=1$; $p=0.560$) in the overall isolation rate of mycobacteria between the two communities. However, five of the seven *M. bovis* were from people living in the rural area (Table 3.14).

Regarding the effect of type of housing as a risk factor for acquiring mycobacterial infection, data collected indicates that the isolation rate of mycobacteria from cases living in good housing was 39.1%, while people living in moderate and poor housing was 23.3% and 21.4% (Table 3.14). This difference was however found to be statistically insignificant ($p>0.05$; $\chi^2=2.36$; $df=2$; $p=0.308$).

A total of 111 cases residing in the rural areas were grouped according to their occupations as follows; 30 and 45 were entirely involved in crop and livestock farming respectively, while 36 were engaged in both activities. The isolation rate of mycobacteria compared among different occupations was found to differ significantly ($p<0.001$; $\chi^2=20.0$; $df=2$; $p=0.0001$) (Table 3.14). Furthermore, *M. bovis* was more prevalent in people with contact with animals.

Table 3.14. Analysis of risk factors associated with cases of tuberculosis in man.

Risk parameter	No. of cases	% positive	Mycobacteria isolated		
			<i>M. tb</i>	<i>M. bovis</i>	MOTTs
1. Age (n=129)*					
Children <16 yrs	34	25.5	4	3	1
Adult 16-50 yrs	88	28.5	20	1	4
Old >50 yrs	7	71.4	4	0	1
2. Sex (n=147)					
Female	67	25.3	13	3	1
Male	80	33.8	18	4	5
3. BCG vaccination (n=99)					
Vaccinated	60	28.3	9	4	4
Non-vaccinated	39	25.6	8	0	2
4. Residence (n=149)					
Urban	28	25.0	5	2	0
Rural	121	30.6	26	5	6
5. Housing (n=108)					
Good	23	39.1	8	1	0
Moderate	43	23.3	7	0	3
Poor	42	21.4	5	1	3
6. Main occupation (n=111)					
Crop farming	30	20.0	4	1	1
Livestock keeping	45	17.8	4	2	2
Mixed farming	36	61.1	15	4	3
7. Drink fresh milk (n=74)					
Yes	41	31.7	9	1	3
No	33	18.1	3	0	3
8. Take sour milk (n=97)					
Yes	92	25.0	16	1	6
No	7	40.0	2	0	0
9. Share household utensils (n=107)					
Yes	102	27.5	20	2	6
No	5	0.0	0	0	0

* Number of cases with the record of the variable.

As it was assumed that mycobacteria excreted from infected cattle is likely to be acquired by man through the consumption of milk and its products, data on the consumption of fresh milk and sour milk as a factor in the spread of disease is summarised in Table 3.14. While 41 of 74 cases indicated that they had taken fresh milk, over 90% of cases had consumed soured milk. *M. bovis* was recovered only from these cases (Table 3.14).

Regarding transmission from man to man by the indirect contact through sharing of utensils, the data showed that it is a common practice to share various utensils with family members. There were no mycobacteria isolated from the five cases which indicated that they did not share utensils. In view of differing sample sizes the difference between isolation rate was found to be insignificant ($p>0.05$; $\chi^2=0.76$; $df=1$; $p=0.382$).

3.3. DISCUSSION

3.3.1. BOVINE TUBERCULOSIS IN CATTLE

The total number of cattle examined in the course of this study, 6383, represented about 1.3% of the total cattle numbers grazing on the Usangu Plains (450,000). Due to the abundance of grazing and the presence of the Ruaha river within the plains, the population of cattle grazing on the plains normally increases during the dry season leading to extensive mingling of the herds from within and outwith the plains. There is also an increased likelihood of cattle mixing with wild animals grazing in the Ruaha National Park in the northern part of the plains.

The presence of tuberculosis cases in the Usangu Plains has been recorded for many years (Markham 1952; Maiseli *et al.*, 1987). In absence of control measures, the situation has not changed. In the current study, a total of 911 (14.3%) of the total cattle examined were classified as reactors by a severe interpretation (Lesslie and Herbert 1975). Using a standard interpretation (i.e. a bovine bias of $>4\text{mm}$) as described by Lesslie and Herbert (1975), the percentage of cattle classified as

reactors dropped to 7.4%. The former interpretation was preferred as the disease was confirmed further by both post-mortem and bacteriology (Tables 3.1.9 and 3.1.10). Overall, the results of this study were higher than in previous studies by Markham (1952) and Maiseli *et al.* (1987) who found the average reactor rate of cattle in Southern Highlands to be 11.3% and 2.0% respectively. The reason for the lower isolation rate in Maiseli's study may have been attributed to the fact that these workers sampled mainly grade or exotic breeds kept on government farms as opposed to the indigenous breed (Short Horn Zebu) sampled in this study and that of Markham (1952). According to Thrusfield (1986), stratification of animals to herds during sampling enables an accurate assessment of the disease in the population. This fact was taken into consideration in the analysis of the results of the current study. 51% of the 239 herds examined had reactor cattle which indicated how wide spread is the disease in the Southern Highlands of Tanzania. In a review by Francis (1947), it was shown that a strong positive correlation does exist between the percentage of infected cattle or herds and the size of the herds from whom reactor cattle were found. This observation was reiterated in the current study, where correlation coefficients of 0.85 and 0.58 were obtained when herd infection and reactor cattle were compared to the size of the herds. Markham (1952) found that every herd with more than 20 head of cattle was infected in the Usangu Chiefdom. The current study did not differ significantly from this trend as 75 to 93.3% of the herds with an average size of more than 20 cattle contained reactor animals. In Nigeria, Shehu (1988) found that all the herds with more than 30 head had reactor animals. These observations are in contrast to that reported in Europe in 1930-50s, where there was a peak of infection among herds of 20 to 30 head and after which a slight decline was noticed (Francis 1947).

Regarding the geographical distribution of herds with reactor cattle, the present study has supported the earlier studies (Markham 1952; Maiseli *et al.*, 1987). The major part of the Usangu Plains involved in the present study included Mbarali sub-district which during colonial days was known as Usangu Chiefdom. This is an area where Markam (1952) found a higher reactor rate (25%) among cattle. In the present study, only 15.8% of cattle tested in the same area were positive. Cattle tested outwith

Mbarali sub-district (Uyole, Makambako and Njombe) had a significantly lower ($p<0.0001$) cumulative reactor rate (7.0%), but this figure was higher than that of Markham (1952) and indicating that the disease could be spreading slowly to other districts outside the Usangu Plains. The reason for the high concentration of bovine tuberculosis cases in the Usangu Plains, particularly in Mbarali sub-district, has yet to be investigated.

One of the aims of the present study was to assess the zoonotic implication of *M. bovis* infection in cattle belonging to pastoralists of the Matebete village. A detailed analysis of the twelve positive herds kept by pastoralists at Matebete village found that the mean reactor rate per herd was 28.5% (range 8.8 to 53.6%) which was double the average reactor rate (14.3%) for the entire plains. Possible explanations for this high rate could be, first, since the residents of this village are mainly Masaai, they are likely to be moving their herds extensively across the plains and, in due course, mix with other herds and wild ruminants. Second, the Masaai are well known to be involved in cattle rustling and thus their herds are unlikely to be stable as there is constant moving in and out of cattle, which may introduce infectious diseases such as tuberculosis.

According to O'Reilly (1995) the specificity and sensitivity of tuberculin tests should be established in each country or geographical area. This suggestion was taken into consideration in the execution of the present study. Although the sample size used to determine sensitivity of the test was small, it was established that the tuberculin test had a sensitivity of 75%, which is slightly lower than other published reports (Monaghan *et al.*, 1994; Table 1.7), but higher than 68.6% reported by Francis *et al.* (1978). However, this figure could be an underestimation, as van Waveren (1954) indicated, in the Netherlands, the percentage of confirmed *M. bovis* cases was increased by 15% when 20 guinea pigs were inoculated with materials from non-visible reactors. A specificity of 100% recorded in the present study was similar to that reported in Ireland (O'Connor 1986) and higher than that reported in Australia (Francis *et al.*, 1978).

The tuberculin test, as employed during the course of the present study, utilised avian and bovine PPDs. Plum (1931) and Stenius (1938) initiated the comparative test in a

bid to distinguish cattle infected with bovine tuberculosis and cattle sensitised by *M. paratuberculosis* and *M. avium*. Non-specific reaction could also be caused by other bacteria, particularly *Norcadia* spp., *Actinobacillus* spp., *Corynebacterium pyogenes*, etc. (Karlson 1962). Although none of these conditions has been demonstrated in cattle in the Usangu Plains, 329 (5.1%) cattle were more reactive to avian PPD than to bovine PPD. This low level of non-specific infection is comparable to that experienced in Karamoja cattle in North East Uganda (Pritchard *et al.*, 1975).

In the course of the current study efforts were made to assess the effect of some physiological parameters on the reactivity of cattle to the tuberculin test. Older cattle were found to be significantly ($p < 0.001$) more affected by bovine tuberculosis than younger cattle, this finding is in agreement with other studies showing that older cattle were more affected than younger cattle (Stamp and Wilson 1946; Shehu 1988). It has been argued that this disparity is mainly due to increased chances of exposure rather than slow progression of disease (Stamp and Wilson 1946; Stamp 1948), and that younger stock are equally able to mount measurable immune responses to tuberculin even as early as the first week of life (Woodward *et al.*, 1979). The preponderance of the disease among the older stock, has its impact on the epidemiology and public health implication of the disease. In pastoralist communities, it is the older cattle which are normally disposed for purposes including sale for money, slaughter for festivals, paying of dowry, etc.. These practices could facilitate the spread of the disease not only to other cattle but also to man when animal products are consumed without proper treatment.

A report by Carmichael (1938) has indicated differing prevalence of disease among the different types of Zebu Cattle. Zebu cattle found in the Western Uganda, North-west Tanzania and Eastern Zaire are those with enormous spreading horns, generally larger and "leggier" in appearance (Ankole Zebu cattle). These cattle were more affected by tuberculosis, while those found in most parts of Tanzania and Kenya which are stocky, short horned and have an appreciable hump, were regarded to be resistant (Hornby, 1934). In the present study a comparison made between indigenous cattle and exotic breeds showed that the reaction of Short Horn Zebu cattle to tuberculin was significantly higher than exotic cattle (*Bos taurus*) ($p < 0.05$).

This could be attributed to the different management systems rather than the breed variation because, the presence of tuberculous lesions and isolation of *M. bovis* from zebu cattle demonstrated that *Bos indicus* are susceptible to infection. In the Usangu Plains, exotic breeds are either kept by government farms or by progressive small dairy holders. These animals are normally kept under a semi-intensive management system, and have minimal movement within the plains. They, therefore, have less contact with indigenous cattle thus minimising their chances of becoming infected. This finding is supported by Shehu (1988), who found that, in Nigeria, indigenous cattle kept under nomadic management system were more affected than those kept under intensive management system (13.7% vs 0.0%) and attributed the difference to the extent of mixing of cattle from different herds and different geographical areas.

In earlier studies carried out in Nigeria (Shehu 1988) and in Tanzania (Markham 1952), assessment was made on the effect of sex on tuberculin reactivity of cattle. Markham found more male than female cattle reacted to tuberculin (14.4 vs. 7.7%) in areas where male cattle are used for draught power. Shehu (1988) did not observe any sex preference of the disease (11.4% male vs 11.9% females) whereas in the present study, a significant difference was found between male and female cattle reacted to tuberculin ($p < 0.05$). It could be reasoned that more adult cattle were male than female, but the results of the present study found that 68.4% of older cattle were male reactors compared to 58.4% female. The simpler explanation of this feature could be that male cattle are more likely to move from one herd to another either for breeding or for draught work with neighbours' or relatives' herds. Since it was speculated that the preponderance of the disease among male cattle could be due to their activity, a comparison between bulls and castrated cattle was made and found that reactivity to tuberculin was significantly higher in castrated cattle ($p < 0.01$). Castrates are mostly used for ploughing fields during the rainy season. They are normally kept for longer in the herd than bulls and bullocks, which are sold either at an early age or after their effective years of service.

It has been established that cows are anergic a few weeks post-partum (Kerr *et al.*, 1946a; O'Reilly 1987). The current study was in disagreement with this suggestion, as a significantly higher ($p < 0.05$) reactor rate was found among lactating than non-

lactating animals, however, this difference could be attributed to the analysis made on all the nine months post-partum. Analysis of periods of three, six and nine months post-partum showed that cows were reacting poorly during the first three months post-partum as compared to above three months (data not shown). There was 18.1% compared to 6.7% reactors among older (≥ 4 years) and younger (< 4 years) lactating cattle. This difference was found to be statistically significant ($p < 0.01$; $\chi^2 = 7.69$; $df = 1$; $p = 0.0055$).

The study at Matebete village was designed to provide knowledge on the effect of human tuberculosis to infection in cattle and *vice versa*. The present study showed that cattle owned by families with a history of tuberculosis had a significantly higher ($p < 0.001$) reactor rate (31.4%) compared to 14.0% found in herds owned by the families with no history of tuberculosis. This finding is in agreement with a recent study in Monze district, Zambia, where Cook and colleagues (1996) found that there was an increased risk ratio of having cases of human tuberculosis when families are in contact with positive herds of cattle ($RR = 6.19$).

A preliminary survey, based on existing data of slaughter slab condemnations in Mbarali sub-district, showed that up to 20% of slaughter cattle had tuberculous lesions (Kazwala *et al.*, 1993). The present study reaffirmed that observation, where it was found that 26.8% of cattle had tuberculous lesions. This figure is also comparable to other early studies in the same area, where Markham (1952) found that 23.3% of 335 cattle examined had tuberculous lesions at post-mortem. Similar studies on Zebu and Ankole cattle in Uganda by Carmichael (1938) found a lower number (0.8%) of Zebu cattle having tuberculous lesions, while 17% of Ankole cattle had tuberculous lesions. Subsequent to this finding it was speculated that Zebu cattle are less susceptible to tuberculosis (Carmichael 1938). This notion is in disagreement with the present study as well as studies carried out in recent years in Uganda, which found a higher incidence (27%) of tuberculous lesions amongst Zebu cattle (Pritchard *et al.*, 1975), disproving the earlier proposition that Zebu cattle are resistant to tuberculosis.

According to Carmichael (1938), tuberculous lesions in Zebu cattle are small, caseous tending towards calcification and are confined to the thoracic glands. This

observation was confirmed by the present study where tuberculous lesions were more prevalent in bronchial and mediastinal lymph nodes as well as in the lung tissue. This finding confirmed that the primary focus in bovine tuberculosis in cattle is the respiratory tract (Stamp 1946;1948). Involvement of retropharyngeal (28.9%) and mesenteric (15.5%) lymph nodes indicates that the alimentary route is also important. Although the majority of cattle in the Usangu Plains are grazed extensively during the day time, opportunities for close contact do occur. They congregate at watering points and are confined within kraals at night, hence cattle to cattle transmission of infection is likely to occur in a similar manner to cattle kept under intensive management systems. In Australia, Leeper and Pearson (1973) found that the management system influenced the type of lesions encountered at slaughter. Cattle grazed on the range had predominantly thoracic lesions as compared to abdominal lesions (38.0% vs. 1.8%), whereas those on improved pasture had lesions evenly distributed between the thorax and abdomen (15.3% vs. 16.6%). Although the vegetation on the Usangu Plains is mainly short grass, however, mountain grass is found on the peripheral areas of the plain. This is due to an increase in the altitude on escarpments of the Rift valley. It can be speculated that the difference in climate might be one of the reasons for the disparity of percentage of cattle with tuberculous lesions as well as reactor cattle examined in Mabarali sub-district and Njombe district. The effect of climate and cattle management systems on the prevalence of the disease in the Usangu Plains might need further investigation.

In countries like Tanzania, where there is no routine screening of cattle using the tuberculin test, the data on bovine tuberculosis is acquired from abattoir returns. The accuracy of abattoir returns is dependent on the competence of the meat inspectors. One of the challenges to meat inspectors' diagnosis of bovine tuberculosis experienced in the present study was due to the presence of granulomatous lesions on the surfaces of the thoracic segment of the aorta. Since the right bronchial lymph node is so close to the aorta it is very likely that lesions on the two organs could be confused. There was a total of 199 carcasses with such lesions. Similar lesions have been found elsewhere in the country (Mtei and Sanga 1990) and are believed to be due to *Onchocerca armillata* infestation.

In order to increase the sensitivity of post-mortem inspection, Corner *et al.* (1990) utilised an enhanced meat inspection procedure. In the present study this procedure was employed and found to increase the detection rate from 8.0% to 26.8%. This finding is in line with that of Corner *et al.*, (1990), who revealed that 47% tuberculous lesions had been missed previously at routine meat inspection.

Post-mortem findings were intended to assess the reliability of the tuberculin test in detecting lesioned animals, a key factor when conducting voluntary control programme by test and slaughter. As has been indicated by Wilesmith and Williams (1986), in many control programmes there will be herds with reactors where subsequent post-mortem and laboratory examinations fail to confirm infection. It appeared in the current study that using the enhanced post-mortem procedure, the positive predictive value of the tuberculin test was 70% while using routine post-mortem procedures the positive predictive value dropped to 25.3%. These figures are relatively lower than the 88% deduced from results obtained by Markam (1952). There were many lesion positive cattle which could not be classified as reactors by the present skin test (Table 3.9). These lesions could be due to non-tuberculous mycobacteria or other micro-organisms (Karlson 1962), although the comparison of tuberculin reaction due to either avian or bovine PPDs in cattle with tuberculous lesion showed that cattle reacted more to bovine PPD than avian PPD (Figure 3.3). It should however, be noted that reactions to avian PPD indicated the relative influence exerted by other micro-organisms to the diagnosis of bovine tuberculosis by tuberculin test in the Usangu Plains.

According to one of Koch's postulates, the clinical diagnosis of a disease can only be confirmed if a positive isolation of the causative agent is made from clinical material of affected individuals (Thrusfield 1986). During the course of the present study *Mycobacterium tuberculosis* complex (MTBC) organisms were cultured from all types of specimen collected from the Southern Highlands and from Arusha, thus confirming diagnosis made using tuberculin test as well as post-mortem examination. The initial identification was confined to MTBC as a group because the SUA laboratory performed only few tests, which were not adequate to differentiate isolates to the species level. The overall isolation rate of mycobacteria from cattle lymph

nodes was 4.3%. This figure is relatively lower than that reported by other studies (Rivas *et al.*, 1985; Huchzermeyer *et al.*, 1992). The frequency of isolation of *Mycobacterium* spp. showed that 80% and 29.7% of positive samples collected from Arusha and the Usangu Plains, respectively, were due to *M. bovis*. Interestingly, one strain was identified as *M. tuberculosis* at SMRL. Isolation of *M. tuberculosis* from cattle tissues has also been recorded in Kenya (Waddington 1965).

The high proportion of isolations of atypical mycobacteria support the view that non-specific reactions exhibited by cattle from the Usangu Plains were actually due to environmental mycobacteria. This finding emphasises further the need to use a single comparative tuberculin test (Ritchie 1953; Lesslie and Herbert 1975) when screening cattle for bovine tuberculosis in Tanzania.

The isolation of mycobacteria from milk samples collected from the Usangu Plains revealed that 31 (3.9%) of all samples were positive. Of those, only two were later identified as *M. bovis*, the rest were atypical mycobacteria. In comparison to some published reports, this isolation rate is lower than that reported in India (Gajinder and Sengupta 1957), Iraq (Kastandi *et al.*, 1989) and Nigeria (Okolo 1992), where *M. bovis* was encountered in more than 50% of the positive milk samples from cases of tuberculous mastitis. In agreement with the findings of the present study, lower proportion of *M. bovis* in mycobacteria positive milk samples has been reported in other studies as well (Appuswamy *et al.*, 1980; Gertman *et al.*, 1990; Yehualaeshet 1995). It should be noted that in the current study, milk samples came from cattle which had no known history of tuberculous mastitis and all except one had reacted negatively to the tuberculin test. Although the isolation rate of *M. bovis* from milk samples analysed in this study was relatively low, its public health implications should be taken seriously. Kleeberg (1984) has indicated that one cow can excrete enough viable bacilli to contaminate the milk of up to 100 cows, when their milk is mixed.

Samples of milk collected from cattle belonging to pastoralists residing in Matebete Village yielded a high proportion (19.2%) of atypical mycobacteria and similar species of mycobacteria were isolated from human cases from the same area, suggesting a common source of infection. However, isolations of atypical

mycobacteria from milk samples might have been due to contamination from the surfaces of the udder, as cleaning of the udder was rarely performed by pastoralists during milking

3.3.2. IMPORTANCE OF *M. BOVIS* INFECTION IN MAN

In Tanzania, infection caused by *M. bovis* in man has been regarded so far as of no significance to human health (NTLP Report 1987). This notion emanated from the lack of isolations of the organism from clinical specimens collected from cases of pulmonary tuberculosis. As indicated by Pritchard (1988), the medical profession in many countries has failed to appreciate the contribution of *M. bovis* infection to tuberculosis morbidity and mortality because routine typing has seldom been so extensively carried out in man. So far, there have been no concise studies carried out in Tanzania to specifically identify *M. bovis* as a cause of human tuberculosis. One relevant study (Richter *et al.*, 1991) examined a total of 271 cases of extra pulmonary tuberculosis diagnosed at Muhimbili Medical Centre, Dar-es Salaam and were able to isolate mycobacteria from 6% of the samples, but could not speciate the strains. In the current study, 29.5% of 149 human specimens collected from Arusha, Mbeya, Iringa, Dar-es Salaam and Morogoro Regions were found to be mycobacteria positive. This figure is lower than that recorded in another Tanzanian study (EA/BMRC 1975), where 48 to 55% of specimens collected from patients attending tuberculosis clinics in various districts were positive for mycobacteria. The study did not specify the species involved. The results (Table 3.12) indicated that a higher proportion (39.6%) of mycobacteria was recovered from extra-pulmonary specimens than from pulmonary cases (24.0%) ($p < 0.05$) and that there was a significantly higher ($p < 0.001$) isolation rate among specimens collected from Arusha compared to those collected from other parts of the country. Similar observations were recorded in studies conducted in Nigeria, which found an uneven distribution of mycobacterial infection between the north and south of the country (Alhaji and Schnurrenberger 1977; Idigbe *et al.*, 1986).

Overall, of the 44 strains recovered from both forms of the disease, *M. tuberculosis* was the main isolate (70.5%) which is similar to that reported in the U.K. (Wilkins

and Roberts 1985). Seven (15.9%) isolates were classified as *M. bovis*, a figure which is comparable to those recorded in many other studies (Table 1.9 - Chapter 1), where the proportion of *M. bovis* ranged from as low as 0.004% in Canada (Liss *et al.*, 1994) to as high as 22.8% in Russia (Kovalyov 1989). *M. terrae* was found in four cases (9.1%), while there were single strains of each of *M. avium* (2.3%) and *M. fortuitum* (2.3%). Apart from *M. avium*, the pathogenic importance of the other strains of atypical mycobacteria isolated in this exercise have not been recorded elsewhere. Barrera and de Kantor (1986) in Argentina and Idigbe *et al.* (1986) in Nigeria isolated several species of atypical mycobacteria from human cases of pulmonary tuberculosis, similar to the current study.

A comparison of the proportion of *M. tuberculosis* complex species isolated from the extra-pulmonary and pulmonary forms of the diseases showed that there was no significant difference in isolation of *M. tuberculosis* from either form of the disease. In contrast, *M. bovis* was seven times more frequent amongst mycobacteria from extra pulmonary compared to those from pulmonary cases (28.6 vs. 4.3%). Although the number of isolates is small, this observation is similar to that experienced in European countries at the start of the bovine tuberculosis eradication programme (1930-50's), when the isolation rate of *M. bovis* from extra pulmonary was five times higher than from pulmonary cases (Torning 1965; Kleeberg 1984). However, this picture has changed in recent years in developed countries (Grange and Yates 1994). Studies in Argentina and in the Republic of Ireland have shown that the proportion of *M. bovis* in bacterial positive pulmonary cases was higher than in extra pulmonary cases (Barrera and de Kantor 1986; Cormican and Flynn 1992; Cotter *et al.*, 1996). The likely explanation for this difference, as put forward by Kleeberg (1984), is that in a situation where tuberculosis is widespread, infection due to *M. tuberculosis* may have prevented super-infection with bovine tubercle bacilli, since the first infection will give rise to a measure of immunity against the second. Consequently, in a situation where *M. tuberculosis* is in decline, *M. bovis* infection is likely to increase in people in high risk occupations, such as livestock keepers (Barrera and de Kantor 1986; Badalik *et al.*, 1995) or abattoir workers (Robinson *et al.*, 1988).

As part of the study at Matebete Village, sputum was collected from 20 suspected cases of human tuberculosis from 12 families who agreed to participate in this study. From these specimens, the two *M. tuberculosis* isolates were from families identified as YM and PS with herds having reactor rates of 41.7 and 22.7 percent. There were two isolates of *M. terrae* which came from members of the same family (MGK), whose herd had a reactor rate of 20.2% (Table 3.5.). A few sputum specimens were collected from two other villages in the Usangu Plains viz. Muungano and Upagama (Table 3.4). These villages had several herds of cattle tested and found to be positive. However, none of the human specimens yielded mycobacteria.

Of the twelve families residing in Matebete village, whose cattle were tested, two had no history of tuberculosis and the cattle they owned had a significantly lower ($p<0.01$) reactor rate compared to those families which had a history of tuberculosis. In view of the fact that the families with history of tuberculosis had high cattle reactor rate, while those without a history of tuberculosis the reactor rate was lower and that mycobacteria was isolated from families with tuberculosis. These findings underline the relative importance of cattle as a source of infection to man and *vice versa*.

Atypical mycobacteria were isolated from milk samples of cattle belonging to individuals from whom *M. terrae* also was isolated, which may indicate that cattle might be a source of not only pathogenic mycobacteria but also atypical mycobacteria to man or both cattle and man are infected by mycobacteria from the environment. Similar observations have been made in other African countries. For example, in a study in Nigeria, Shehu (1988) cultured sputum from cattle keepers and milk from their cattle and was able to isolate atypical mycobacteria from four of 29 sputum samples and one out of 90 milk samples collected. Therefore, the study at Matebete village has highlighted the significance of mycobacterial infections for the human population from in contact infected cattle.

3.3.3. ASSESSMENT OF RISK FACTORS

Information gathered using the questionnaire was able to provide further knowledge on other risk factors associated with the occurrence of tuberculosis in man.

In an earlier study carried out in Tanzania (EA/BMRC 1975), it was found that more younger people (<35 years) were affected by both pulmonary and extra-pulmonary tuberculosis than older people. In the current study there was no significant difference ($p>0.05$) in the isolation rate of mycobacteria amongst different age groups, despite the fact that the percentage of culture positive cases was higher (57.1%) in older people above 50 years of age. However, there were proportionally more *M. bovis* isolated from children under 15 years than in other age groups. This finding necessitates a need for a further investigation on the preponderance of *M. bovis* infection in children in Tanzania. It has been reported elsewhere that children are more likely to be infected by *M. bovis* than other age group especially in societies where raw milk is consumed (Kleeberg 1984). The preponderance of *M. bovis* in younger age groups as seen in this study is in agreement with many of the findings recorded in developed countries prior to control of disease in cattle (Griffith 1937; Kleeberg 1984; Wilkins and Roberts 1985). However, in recent years, this pattern has changed in developed countries, where *M. bovis* largely occurs due to recrudescence of infection in old people rather than new infections in the younger generation (Grange and Yates 1994).

There was no sex variation amongst patients from whom mycobacteria were isolated ($p>0.05$). Of the seven *M. bovis* strains isolated in this study, three were from female and four were from male cases. This finding is in agreement with observations made in England in 1930's where the male to female ratio was 6:5 (Griffith 1937). However, it is in contrast with more recent reports in Canada (Wigle *et al.*, 1972) and in UK (Yates and Grange 1992) where more women were affected than man (ratio ~2.12:1). In all referred situations, no explanation for the sex difference in disease pattern was given.

The effectiveness of BCG vaccine in protecting human population against tuberculosis in the developing world has been reported to vary from 0 to 80% (Murray *et al.*, 1990). This relative ineffectiveness of BCG vaccine has been confirmed recently in Malawi (Karonga Prevention Trial Group 1996). The results of the present study seem to concur with these findings, as there was no significant difference ($p>0.05$) between culture positive patients who were vaccinated and those

which were not vaccinated at childhood. Interestingly, the four *M. bovis* isolates were from cases which had had BCG vaccination. It should be noted that BCG vaccine was originally made from *M. bovis* strain (Larson and Evans 1929), which was passaged and found to lose its pathogenicity to man but maintained its immunogenic properties (Bloom and Murray 1992).

It is assumed that living in rural areas exposes people to many zoonoses as contact with animals is more frequent (Wilson 1954). In the present study, no significant difference ($p < 0.05$) was observed in the isolation rate of mycobacteria among patients living in the rural and urban areas. It can be speculated that the migration of individuals from rural to urban areas, coupled with uncontrolled livestock keeping in towns and cities in Tanzania, could lead to equal exposure to many zoonoses including tuberculosis. However, five of the seven strains (71.4%) of *M. bovis* were from patients from rural areas. This latter finding is in agreement with many of the studies carried out in Europe in 1930s to 50s (Griffith 1937; Sigurdsson 1945), and more recently in Slovakia (Badalik *et al.*, 1995). In those studies, there was a clear difference in the levels of *M. bovis* infection between the rural and urban populations, with the former being more affected.

In view of the above observations, an attempt was made to establish if an individual's occupation influenced the occurrence of the disease. According to the results of the present study, one *M. bovis* isolate (3.3%) came from a patient who was working solely on crop husbandry and no contact with cattle. There were two (4.4%) and four (11.1%) cases of *M. bovis* from patients who were solely involved with cattle keeping and those who were involved in both cattle keeping and crop farming respectively. The findings of the present study are in agreement to those found by Cutbill and Lynn (1944) in U.K., where 16.4% compared to 1.6% of *M. bovis* isolation rates were from cattle-contact and non-cattle-contact groups respectively.

The transmission by direct transfer of bovine tubercle bacilli from cattle to man via the respiratory route is likely to have occurred in only one case encountered in this study (from the Usangu Plains) as opposed to many cases which were encountered in earlier studies (Cummings 1933; Cutbill and Lynn 1944) and in recent studies involving *M. bovis* from other animals (Robinson *et al.*, 1988; Fanning and Edwards

1991; Dalvisio *et al.*, 1992; Stetter *et al.*, 1995). It should be noted that most *M. bovis* recovered from extra-pulmonary tuberculosis cases came from Arusha and the limitation of resources in the execution of this study did not allow collection of specimens of pulmonary tuberculosis from this region. Therefore, a need for further investigation of this parameter is important in any future research on this subject in Tanzania.

The most common route of transmission of *M. bovis* from cattle to man is through ingestion of milk (Kleeberg 1984). It is unfortunate that more than half of the patients were not asked about their eating habits by the medical practitioners and surgeons attending the cases, particularly from Arusha, Dar-es Salaam and Morogoro. The responses were from those cases, which were interviewed by the staff of NTLP in the Usangu Plains, hence an assessment of this factor would not lead to meaningful conclusion. However, single isolates from patients indicating consumption of fresh milk/sour milk confirm the view expressed by other studies (Kleeberg 1984). Milk and milk products, as a source of *M. bovis* infection for man in African societies has been a subject of controversy. Alhaji and Schurrenberger (1977) and Shehu (1988) in Nigeria believed that milk became contaminated with mycobacteria from the milkers or those who processed and marketed the milk rather than endogenous excretion of *M. bovis* from infected cattle. Pritchard (1988), citing the review by Kleeberg (1984) in South Africa, indicated that souring of milk in calabash containers destroys *M. bovis*. This statement contradicts Kleeberg (1984) who indicated that milk products such as yoghurt and cream cheese made from unpasteurised milk have been found to contain TB bacilli up to 14 days after manufacture, and butter as long as 100 days after manufacture.

Assessment was also made of the consumption of meat, offals and blood as risk factors for *M. bovis* infection in man. In Tanzania there are some ethnic groups (particularly Masaai and Barbaigs) which have the habit of consuming raw beef, kidney and liver as well as drinking raw blood from cattle. The results of the present study failed to demonstrate this route of transmission of *M. bovis* to man. However, as indicated by Francis (1973) although there is very small risk from flesh of tuberculous cattle the danger posed to the human population should not be

disregarded on public health and aesthetic grounds. In a study by M'Fadyean (1892), blood from infected cattle was able to cause generalised tuberculosis in guinea pigs. Therefore, a thorough investigation needs to be undertaken to address these animal products as a source of infection to some communities with a custom of taking raw meat and blood.

Griffith and Munro (1935), Cumming and Foster (1933), Cutbill and Lynn (1944) and recently Bouvet *et al.* (1993) and van Embden *et al.* (1994), admit the possibility of human-to-human transmission. In the present study, the possibility of human-to-human infection with bovine bacilli via the oral route was investigated. The data available failed to demonstrate this mode of transmission.

3.3.4. CONCLUSION

In view of the above findings it can be concluded that bovine tuberculosis is endemic in cattle in Tanzania, particularly in the Southern Highlands. Despite the fact that few cattle in the Northern Tanzania had tuberculous lesions, the isolation of *M. bovis* from lymph nodes confirmed the existence of the problem in that part of the country. Furthermore, it can be concluded that *M. bovis* infection does occur in man in Tanzania, and the main manifestation of the disease is the extra-pulmonary form. There is circumstantial evidence that this infection is acquired by man from cattle and the likelihood of the reverse situation is not ruled out.

CHAPTER 4

EVALUATION OF MOLECULAR TECHNIQUES FOR IDENTIFICATION AND TYPING OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX ORGANISMS

CHAPTER 4 EVALUATION OF MOLECULAR TECHNIQUES FOR IDENTIFICATION AND TYPING OF *MYCOBACTERIUM TUBERCULOSIS* COMPLEX ORGANISMS

4.1. INTRODUCTION

The results of the preceding chapter have shown that cattle and man in Tanzania are susceptible to infections caused by species of the *Mycobacterium tuberculosis* complex as well as atypical mycobacteria. In order to demonstrate the zoonotic importance of members of the tuberculosis complex, first, the true identity of the strains recovered from man and cattle should be established and second, the clonal relationship of isolates of the same species recovered from cattle and man should be ascertained. In the previous chapter, conventional techniques for isolation and identification of mycobacteria were employed. The techniques used included microscopy, culture of mycobacteria on Loewenstein-Jensen medium with and without pyruvate added (Jensen 1955; Stonebrink 1958) followed by identification using biochemical tests described by Watt *et al.* (1993). These techniques suffer from low sensitivity [they require at least 10^4 organisms (Yaeger 1967; Bates 1979)], low specificity [inability to differentiate closely related species of the *M. tuberculosis* complex (Prat 1974; Grange 1982; Ratledge and Stanford 1982)] and require more than six weeks to be performed (Shoemaker *et al.*, 1985). An alternative to these techniques would be to isolate mycobacteria using the BACTEC system (Cummings *et al.*, 1975), this system has also been found to be affected by the contaminant present in the specimen (Kirihara *et al.*, 1985). Indirect detection of infection by measuring humoral and cellular immune responses of the affected individual by techniques such as the ELISA, gamma interferon assay, tuberculin test, *et cetera*, also suffers from low sensitivity and specificity (Wood *et al.*, 1992).

The advent of genetic engineering technology has provided microbiologists with alternative approaches to overcome the above mentioned shortcomings. One of the molecular technique which has benefited clinical mycobacteriologists is the polymerase chain reaction (PCR). Polymerase chain reaction as described by Saiki *et al.* (1988) is a primer-directed enzymatic amplification of target DNA carried out *in vitro*. The PCR process as shown in Figure 4.1 has three steps, (i) denaturation of double stranded DNA, (ii) annealing of the primers to the complementary sequence of each single-stranded DNA template, and (iii) primer extension by DNA polymerase along both template strands in opposite overlapping directions with a consequent doubling of the amount of target DNA sequence (cycle 1). This process, if repeated a number of times, results in an exponential increase (2^n - where n is the number of cycles) of up to a million copies of a particular DNA sequence (Figure 4.1). The amplified DNA can be visualised by gel electrophoresis, or analysed further by hybridization or sequencing (Saiki *et al.*, 1986). Since its inception PCR has found numerous applications in research and diagnostics (Coote 1990). These include: i) manipulation of gene sequences, ii) direct sequencing of DNA, iii) identification of gene sequences, iv) detection of low numbers of organisms, and v) evolutionary or epidemiological studies.

In the field of mycobacteriology, the use of the PCR technique has revolutionised the detection and identification of *Mycobacterium* spp. which for long time remained a significant problem.

The importance of molecular typing has emanated from the fact that studies of the epidemiology of tuberculosis either in an outbreak situation or on a pandemic level in both man and animals, have been hampered by the lack of appropriate techniques which could sub-type strains into clusters.

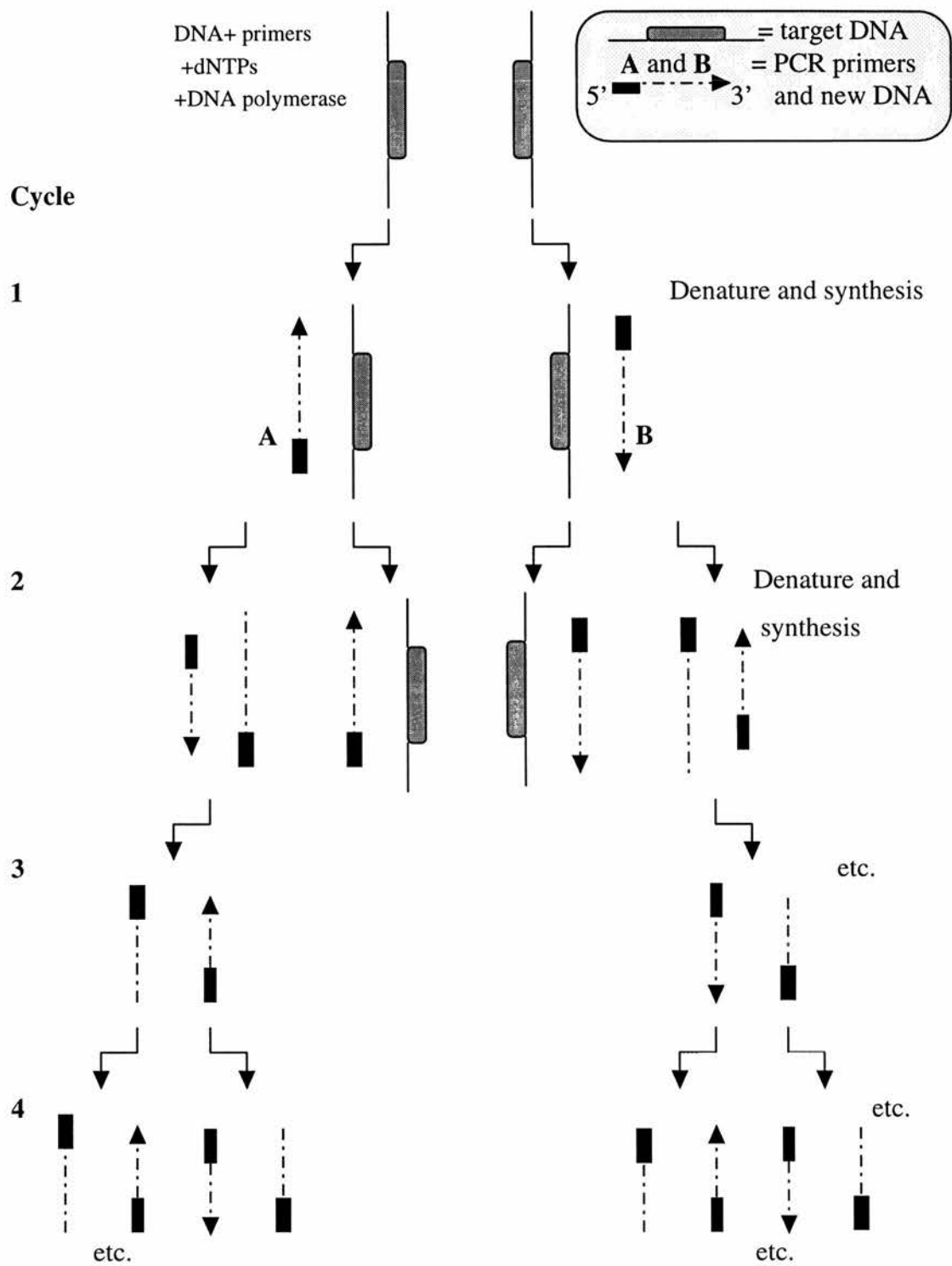


Figure 4.1. The polymerase chain reaction

As indicated in the preceding chapters, the strains belonging to the *M. tuberculosis* complex are too homogeneous and the existing conventional typing techniques which include, serology, phagotyping, antibiotic resistance patterns among others, have either failed to provide polymorphism among unrelated strains or are cumbersome to carry out. The development of molecular typing techniques have provided epidemiologists as well as microbiologists with better tools to understand the dynamics of infections caused by strains of the *M. tuberculosis* complex in man and animals (Crawford 1993; Skuce *et al.*, 1994; van Soolingen *et al.*, 1994a).

Various approaches for detecting and identifying mycobacterial DNA based on PCR have been reported (Hawkey 1994). These approaches include amplification of the genes encoding mycobacterial antigens specific for the taxon *M. tuberculosis* complex as a group or individual species (Table 1.5b) or amplification of specific repetitive sequences such as insertion sequences (Table 1.5a).

Regarding molecular typing, the first DNA typing protocol to be applied was restriction endonuclease analysis (REA). REA was applied to type strains of *M. bovis* from cattle and other animals in New Zealand (Collins and de Lisle 1985). Although the system offered a better typing procedure, it was too cumbersome to be utilised routinely. First, sufficient growth of the cells was required to yield approximately 2-3µg of DNA and second, the DNA fragments produced after digestion with frequent cutting restriction enzymes were too many (>100) to be easily interpreted (Collins *et al.*, 1993). This technique was later improved by Southern blot transfer of the digested DNA fragments onto a membrane and subsequent hybridisation with DNA sequences of IS986 or IS1081 (van Embden *et al.*, 1993; van Soolingen *et al.*, 1993). This latter technique, termed restriction fragment length polymorphism (RFLP) is currently widely used to type strains of the *M. tuberculosis* complex world wide. Repetitive DNA elements commonly used as a probe for RFLP typing of the *M. tuberculosis* complex are presented in Table 1.5a.

Although IS986 would appear to be ideal for typing the *M. tuberculosis* complex strains, strains of *M. bovis* have been regarded to have low copy numbers of IS986 (1 to 6) (Cousins *et al.*, 1993; van Soolingen *et al.*, 1992) thus limiting its typing ability. The use

of IS1081 RFLP as an alternative typing scheme for *M. bovis*, has been limited to its initial studies on *M. bovis* BCG (van Soolingen *et al.*, 1992). The authors found that IS1081 was presented as 5 to 6 copies within the genome of *M. bovis* and *M. tuberculosis* and was integrated at an almost similar spot in many strains regardless of their relatedness, thus greatly reducing its value to discriminate strains. Furthermore, strains of *M. xenopi* also contain this element. The other repetitive DNA elements *viz*, direct repeats (DR), major polymorphic tandem repeats (MTPR) and polymorphic GC-rich repetitive sequences (PGRS) have been regarded to be the best alternative to IS986 typing, however the numerous bands produced by these marker makes the interpretation of results cumbersome (Small and van Embden 1994).

IS986 RFLP typing is now accepted as the standard technique (Hart *et al.*, 1996), and in the standardised RFLP technique as described by van Embden *et al.* (1993), genomic DNA is cut with the restriction endonuclease *PvuII* and utilising a probe derived from the right arm of *PvuII* digested IS986. The resulting fingerprint contains a number of bands which correspond to the copy number of IS986 in an individual strain. This technique provides polymorphic banding patterns in unrelated strains due to the difference in number and position of insertion sequences within the genome of the respective micro-organism.

4.1.1. THE AIMS OF THE STUDY

In the present study, IS986, IS1081, *mtp40* and MPB70 PCR protocols were evaluated for their ability to identify and discriminate mycobacterial strains acquired from a number of countries, including Tanzania. The reason for comparing IS986 and IS1081 was based on the fact that some strains have recently been reported to lack IS986 (van Soolingen *et al.*, 1993), while the use of *mtp40* and MPB70 PCR protocols was to assess their specificity for the test strains listed in Appendix IV. Furthermore, this study would determine the best methods for a further simplification of the PCR protocols to be applied to the strains acquired from the study described in Chapter 3.

Regarding molecular typing, the purpose of this exercise was to evaluate the standard RFLP typing (van Embden *et al.*, 1993), which was going to be applied on strains of *M. bovis* and *M. tuberculosis* acquired from the studies described in Chapter 3.

4.2. MATERIALS AND METHODS

Material and methods for PCR and RFLP typing are described in the General Materials and Methods (Section 2.2.2 - Chapter 2).

4.2.1. BACTERIAL STRAINS

The list of 49 mycobacteria strains used in this exercise is presented in Appendix IV. Seventeen strains from the Netherlands were a kind donation of Dr. J.D.A. van Embden of the Laboratory for Bacteriology and Antimicrobial Agents, and Unit for Molecular Microbiology, National Institute of Public Health and the Environment, Bilthoven. These strains included *M. tuberculosis* strain MT23 a reference strain, which already had been typed using IS986 RFLP by van Soolingen and others (1994b). Tanzanian strains were part of the the first batch of strains submitted to SMRL for quality control checks on the performance of the SUA laboratory. The Australian strains were a kind gift from Dr. D.V. Cousins of Animal Health Laboratories, Dept of Agriculture, South Perth, Australia. The two *M. bovis* BCG were a kind donation by Dr. A.H.J. Kolk of the Royal Tropical Institute, Amsterdam, the Netherlands.

4.3.RESULTS

4.3.2.PCR RESULTS

4.3.2.1. Preliminary PCR results

The expected sizes of the amplification products were 245bp for IS986, 248bp for IS1081, 372bp for MPB70 and 396bp for *mtp40*. These bands were obtained in all PCRs but there were also non-specific bands produced in each of them (Figures 4.2 through 4.5). There was little background amplification products in *mtp40* PCR because this PCR was included in this study at a later stage and hence its reaction conditions were adjusted at its inception, otherwise all other PCR protocols needed to be optimised.

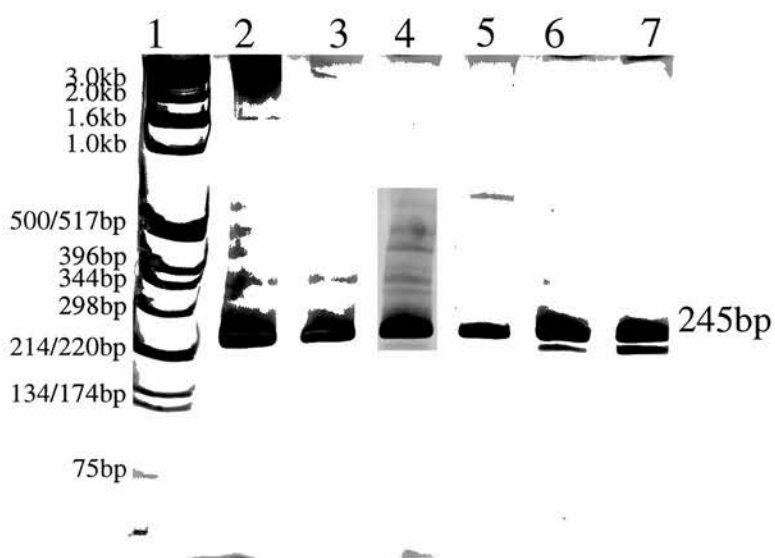


Figure 4.2. Polyacrylamide gel showing amplification products of *M. tuberculosis* and *M. bovis* strains from IS986 PCR described in 2.2.2.3.3.1 (Chapter 2). The desired product is at 245bp. Lane 1 is a molecular weight marker (1Kb ladder), Lanes 2 ,4 and 6 are *M. tuberculosis* strains, Lanes 3, 5 and 7 are *M. bovis* strains. In addition to the 245 bp product, the extra bands are seen in all strains.

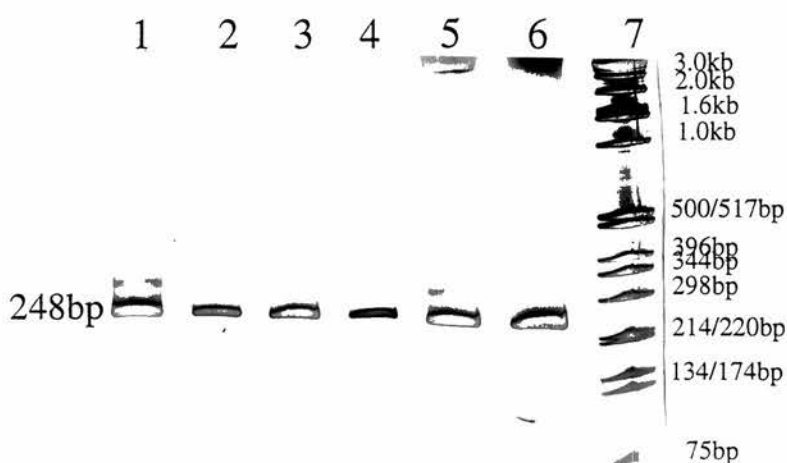


Figure 4.3. Polyacrylamide gel showing amplification products of *M. tuberculosis* and *M. bovis* strains from IS1081 PCR described in 2.2.2.3.3.2 (Chapter 2). The desired product is at 248bp. Lanes 2 ,4 and 6 are *M. tuberculosis* strains, Lanes 1, 3 and 5 are *M. bovis* strains and Lane 7 is a molecular weight marker (1Kb ladder). In addition to the 248 bp product, the extra bands are seen in all strains.

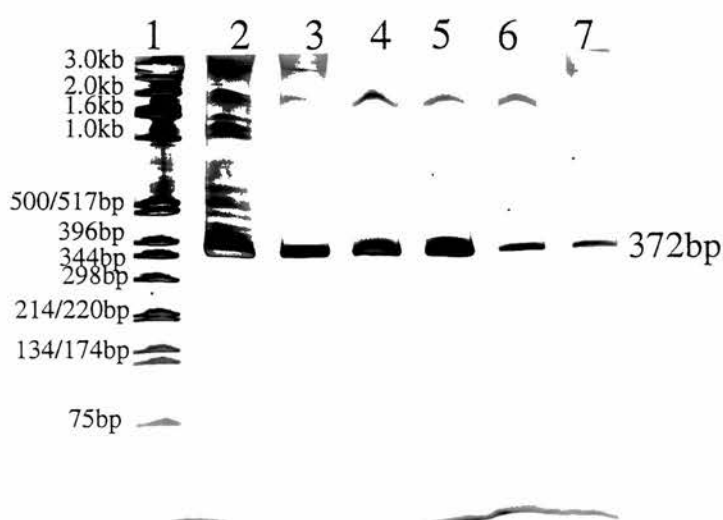


Figure 4.4. Polyacrylamide gel showing amplification products of *M. tuberculosis* and *M. bovis* strains from MPB70 PCR described in 2.2.2.3.3.4 (Chapter 2). The desired product is at 372bp. Lane 1 is a molecular weight marker (1Kb ladder), Lanes 2 ,4 and 6 are *M. tuberculosis* strains, Lanes 3, 5 and 7 are *M. bovis* strains. In addition to the 372 bp product, the extra bands are seen in all strains.

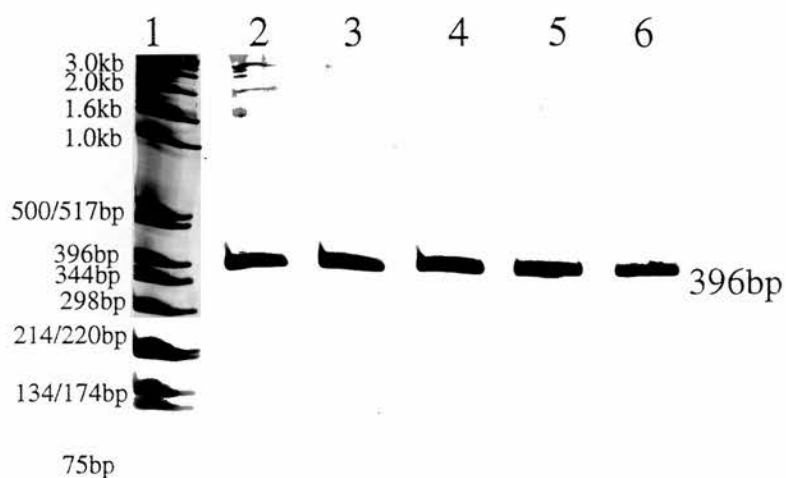


Figure 4.5. Polyacrylamide gel showing amplification products of *M. tuberculosis* strains from *mtp40* PCR described in 2.2.2.3.3.3. (Chapter 2). The desired product is at 396bp. Lane 1 is a molecular weight marker (1Kb ladder), Lanes 2 through 6 are *M. tuberculosis* strains. Since template DNA for this protocols was reduced to 50ng, there was a clear distinction between the desired product (396bp) and few background bands seen above 2Kb mark.

4.3.2.2. Optimisation of PCR

In order to obtain the desired PCR product, attempts were made to optimise PCR conditions whenever it was appropriate. Since PCR is a complex reaction, there are numerous parameters that can be adjusted, however as suggested by Wagner, Jr. and Fowler (1993), the first parameter to be adjusted was annealing temperature by a stepwise increase of 2-3°C, followed by stepwise scaling up and down of the concentrations of primers, *Taq* DNA polymerase, magnesium salt and then template DNA in the reaction..

Optimization experiments on these PCRs revealed that in all cases there was a high concentration of template DNA. Other factors mentioned above were also tested (data not shown) and found not to be the cause of the non-specific amplification shown in Figure 4.2 through 4.5. Experiments on the effect of various DNA concentrations on PCR product were carried out on IS986, IS1081 and MPB70 PCR protocols. In each PCR the following concentration of template DNA were used; 100ng, 50ng, 10ng, 1ng, 100pg, and 10pg. Published protocols indicated that 50ng of template DNA used per reaction. The results of all experiments (Figures 4.6 to 4.8) have shown that the background bands and smearing in the amplicon migration tracts was eliminated when the concentration of template DNA was reduced to between 10 and 100pg.

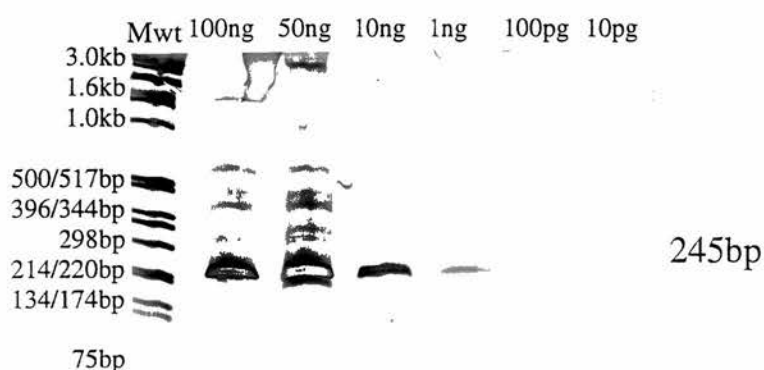


Figure 4.6. Polyacrylamide gel showing the effect of different concentrations of template DNA on amplification of a strain of *M. bovis* (MB27) by IS986 PCR. The desired product is at 245bp. Lane 1 is a molecular weight marker (1Kb ladder), Lanes 2 through 7 are PCR products of 100ng, 50ng, 10ng, 1ng, 100pg, and 10pg of template DNA respectively. Background bands are only seen in Lanes 2,3 and 4 with template DNA concentrations between 10 to 100ng respectively. A single band (245bp) was produced with lower concentrations of DNA (Lanes 4,5 and 6).

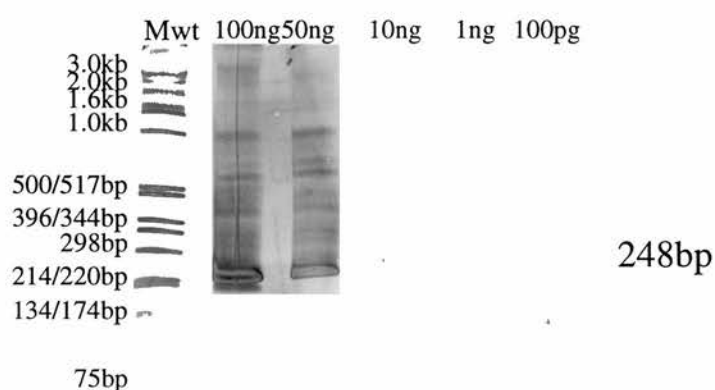


Figure 4.7. Polyacrylamide gel showing the effect of different concentrations of template DNA on amplification of a strain of *M. bovis* (MB27) by IS1081 PCR. The desired product is at 248bp. Lane 1 is a molecular weight marker (1Kb ladder), Lanes 2 through 6 are PCR products of 100ng, 50ng, 10ng, 1ng, and 100pg, of template DNA respectively. Background bands are only seen in the Lanes 2,3 and 4 with template DNA concentration of between 10 to 100ng respectively. A single band (248bp) was produced when 1ng and 100pg of DNA was used (Lanes 5 and 6).

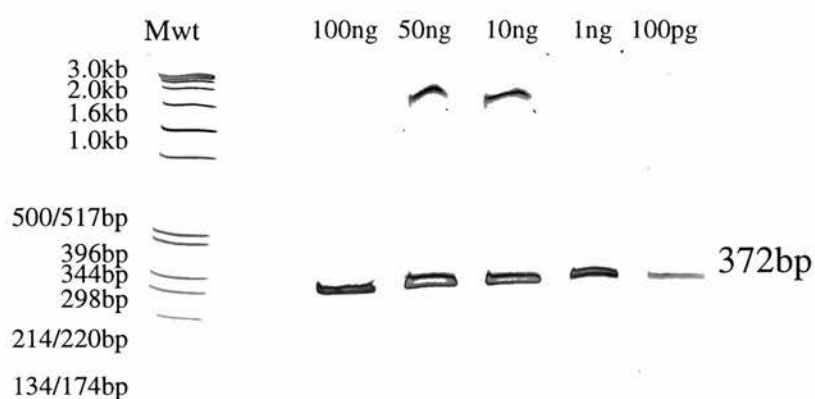


Figure 4.8. Polyacrylamide gel showing the effect of different concentrations of template DNA on amplification of a strain of *M. bovis* (MB27) by MPB70 PCR. The desired product is at 372bp. Lane 1 is a molecular weight marker (1Kb ladder), Lanes 3 through 7 are PCR products of 100ng, 50ng, 10ng, 1ng, and 100pg, of template DNA respectively. Background bands are only seen in the Lanes 3, 4 and 5 with template DNA concentration of between 10 to 100ng respectively. A single band (372bp) was produced when 1ng and 100pg of DNA was used (Lanes 6 and 7).

4.3.2.3. Specificity of PCR protocols

The summary of the results (Table 4.1) of the amplification of all four PCR protocols on 49 test strains shows that all 45 strains of the *M. tuberculosis* complex produced positive results for IS986, IS1081 and MPB70. In addition all 27 Strains of *M. tuberculosis* produced the 396bp band specific for *mtp40*, while none of 14 *M. bovis*, 2 *M. bovis* BCG and 2 *M. africanum* strains produced a *mtp40* PCR product. All 5 MOTTs were negative for IS986, IS1081, *mtp40* and MPB70.

Table 4.1. The results of amplification of 49 strains of mycobacteria using four different PCR protocols.

Taxon	Species	Total positive/total number tested			
		IS986	IS1081	MPB70	MTP40
<i>M. tuberculosis</i> complex					
	<i>M. tuberculosis</i>	27/27	27/27	27/27	27/27
	<i>M. bovis</i>	14/14	14/14	14/14	0/14
	<i>M. bovis</i> BCG	2/2	2/2	2/2	0/2
	<i>M. africanum</i>	2/2	2/2	2/2	0/2
MOTTs					
	<i>M. fortuitum</i>	0/1	0/1	0/1	0/1
	<i>M. marinum</i>	0/1	0/1	0/1	0/1
	<i>M. gordonae</i>	0/1	0/1	0/1	0/1
	<i>M. malmoense</i>	0/1	0/1	0/1	0/1
	<i>M. kansasii</i>	0/1	0/1	0/1	0/1

4.3.3. Molecular typing - IS986 RFLP

RFLP analysis was restricted to only 23 of the 49 strains tested by PCR but included the reference strain MT23, and isolates of both *M. tuberculosis* and *M. bovis* (Appendix IV). The reference strain of *M. tuberculosis* was included in this study as a positive control for the IS986RFLP typing as described by van Embden *et al.* (1993). According to the

fingerprint pattern presented by van Soolingen *et al.*(1994b), *M. tuberculosis* (Mt14323 whose number at MRI is MT23) produced 12 hybridization bands when a 245bp IS986 PCR product was used as a probe. Similar results were reproduced during this study (Figure 4.9. Lane 10), showing that the procedure at MRI could yield results which could be compared with those in the literature. All except one of the Tanzanian strains of *M. tuberculosis* showed a pattern of 10 bands similar to each other but different to the reference strain. One strain, T31, showed a pattern of 16 bands (Figure 4.9. Lane 2) that was different to MT23 and other Tanzanian strains (Figure 4.9.).

Regarding IS986RFLP typing of 10 strains of *M. bovis* and 2 strains of *M. bovis* BCG, a single hybridization band at a 1.9Kb PvuII restriction fragment was found in eleven of twelve strains (Figure 4.10. Lanes 1-6, 8-10, and 16-17). One strain of *M. bovis* (MB10) gave a hybridization product at 2.0Kb mark (Figure 4.10. Lane 18) This strain, together with the two strains of *M. bovis* BCG were from the Netherlands, while the remaining 9 strains originated from Australia.

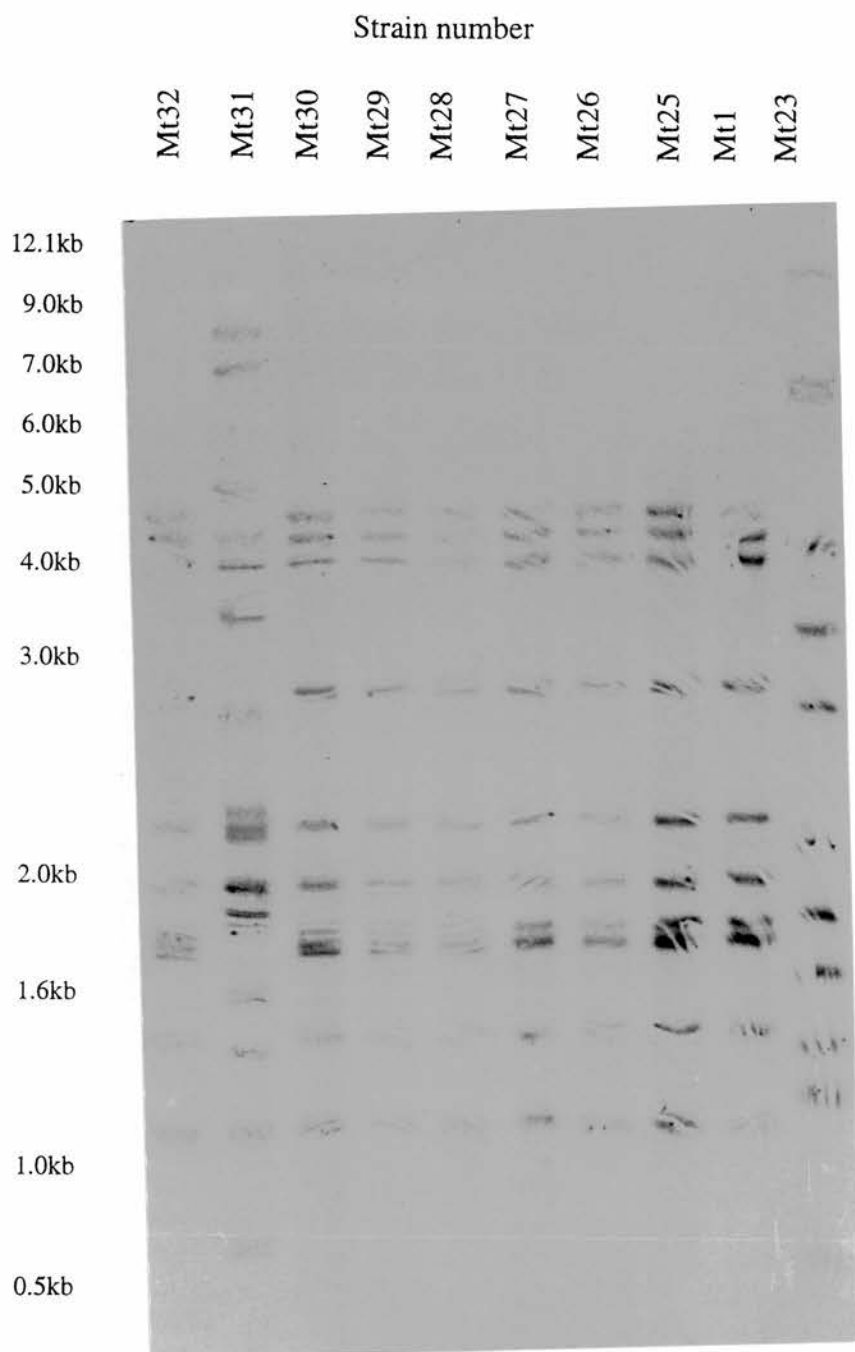


Figure 4.9. IS986 RFLP DNA fingerprint analysis of 10 strains of *M. tuberculosis* cleaved with *Pvu*II and probed with the 245bp IS986 PCR product. Lanes 1 to 9, DNA from *M. tuberculosis* strains originating from Tanzania and Lane 10 DNA from *M. tuberculosis* (MT23) reference strain. Numbers on the left indicate sizes of standard DNA fragments (kilobase pairs).

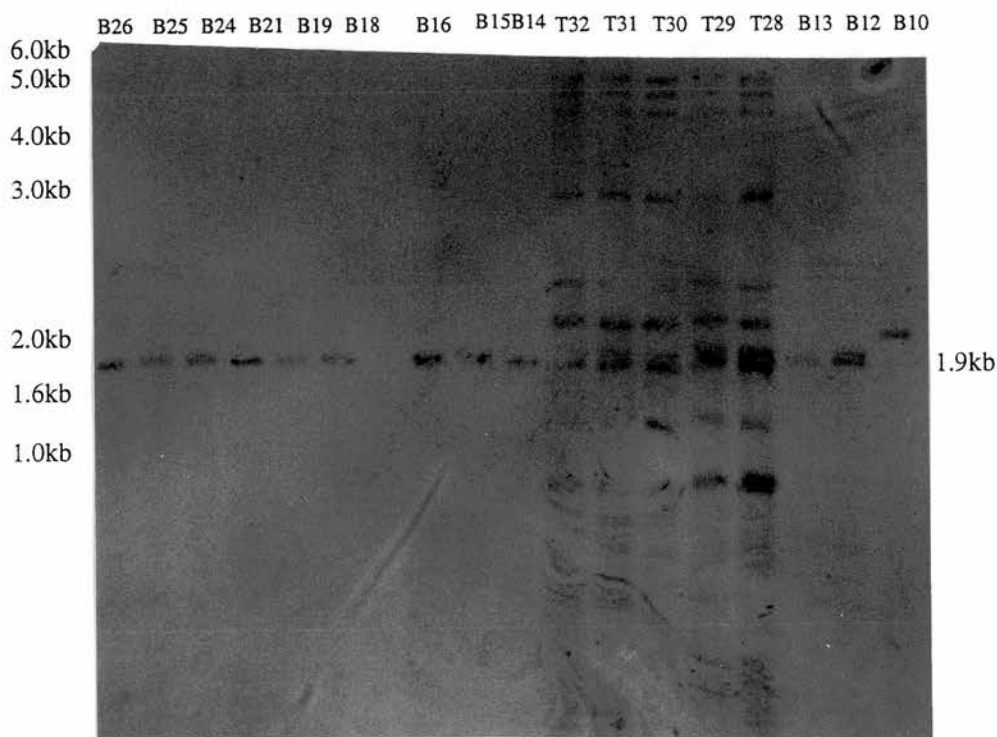


Figure 4.10. IS986RFLP DNA fingerprint analysis of 17 strains of *M. bovis* (n=12) and *M. tuberculosis* (n=5) cleaved with *Pvu*II and probed with the 245bp IS986PCR product. Lanes 1 to 6 and Lanes 8-10, DNA from *M. bovis* strains originating from Australia; Lanes 11 to 15, DNA from *M. tuberculosis* from Tanzania similar to those presented in Figure 4.9 (Lanes 1,3, 6 to 8); Lanes 16 and 17, DNA from *M. bovis* BCG and Lane 18, DNA from *M. bovis* from the Netherlands. Numbers on the left indicate sizes of standard DNA fragments (kilobase pairs).

4.4. DISCUSSION

Epidemiological studies of bovine tuberculosis in man and cattle have never been easy, because of the dependence on cultivation of *M. bovis* and a correct identification of species. For many years isolation of *M. bovis* and other members of the *M. tuberculosis* complex has been made on Loewenstein-Jensen medium with or without sodium pyruvate added, this activity is time consuming. Furthermore, the differentiation of members of this group is very difficult due to the overlapping properties between members of this taxon (Wayne and Kubica 1986). The range of phenotypic differentiation techniques, and a phagotyping technique have been unable to type these strain into related clusters of epidemiological importance (Drobniewski *et al.*, 1994; Saunders 1995; Hart *et al.*, 1996).

Strains of the *M. tuberculosis* complex, recovered from cattle and human specimens (Chapter 3), were isolated and identified using conventional techniques, however it was not possible to link any of the related strains. In order to obtain useful epidemiological information of these infections in man and in cattle it was imperative that the newly developed molecular techniques would need to be employed. In common with any other new technique an evaluation of the DNA based techniques was required prior to their application on the field strains recovered from Tanzania. The molecular techniques for identification and typing of strains belonging to the *M. tuberculosis* complex i.e. PCR and RFLP were respectively evaluated on number of laboratory strains of mycobacteria from various countries including Tanzania.

In the present study, several PCR methods were evaluated. These included IS986 PCR (Hermans *et al.*, 1990), IS1081, modified from van Soolingen *et al.* (1992), MPB70 (Cousins and Wilton 1992) and *mtp40* as described by del Portillo *et al.* (1991). The desired products for IS986 and IS1081 (245bp and 248bp respectively) were obtained from all 44 strains belonging to the *M. tuberculosis* complex and none of the five atypical mycobacteria were positive. As indicated in Chapter 2, the primers for IS1081 as

published by van Soolingen and others (1992) were intended to amplify a 298bp product but unfortunately the PCR was found to generate extra bands (data not shown) and hence a new pair of primers (Collins and Stephen 1991) were designed which amplified a product of 248bp.

In order to detect, specifically, certain species of micro-organisms, several workers have selected DNA sequences which encode antigens that have been used in immunological identification tests for the species (e.g. ELISA). In their initial publication on MPB70 PCR, Cousins and Wilton (1992) indicated that the PCR was specific for *M. bovis* because the primers were designed to amplify a single copy gene encoding the major secretory antigen MPB70 (18 kD antigen). This protein has been recorded to be specific for *M. bovis* when used as an antigen for ELISA (Radford *et al.*, 1988; Wood *et al.*, 1992). However, the results of the present study have shown that MPB70 PCR amplified a product from all 44 strains of the *M. tuberculosis* complex in similar manner to that found by Cousins and Wilton (1992). It therefore can be concluded that while MPB70 PCR may be specific for the *M. tuberculosis* complex it is not specific for *M. bovis* alone. It is believed that the specificity of the MPB70 antigen for *M. bovis* lies on one of its three epitopes (Hewinson R.G. - personal communication 1994). Since the DNA sequence for the epitope claimed to be specific for *M. bovis* has not been published, it was not possible to redesign the MPB70 PCR to amplify specifically *M. bovis* and not other members of the *M. tuberculosis* complex.

The *mtp40* PCR as described by del Portillo and colleagues (1991) was the first PCR protocol reported to differentiate one member of the *M. tuberculosis* complex from the rest. The PCR was designed to amplify a single copy gene encoding an antigen termed *mtp40* reported to be found in *M. tuberculosis* only (Parra *et al.*, 1991). The PCR was adopted as published by del Portillo *et al.* (1991) and was found to be specific to all strains of *M. tuberculosis*. Neither of the DNAs from strains of *M. bovis*, *M. bovis* BCG nor atypical mycobacteria were amplified in the present study which has underlined the importance of PCR in identification of individual strains.

Evaluation of RFLP typing was important as this procedure is greatly affected by variability in electrophoretic, blotting and hybridisation conditions carried out at different laboratories (van Embden *et al.*, 1992; Hart *et al.*, 1996). In the current study one strain of *M. tuberculosis* (MT23) which is the same as *M. tuberculosis* Mt 14323 (van Soolingen *et al.*, 1994) was utilised as a positive control for this study. The RFLP produced by MT23 was exactly the same as that of Mt14323, implying that the RFLP technique performed in the current study was of the same standard as in other reputable laboratories.

In the current study the RFLP typing of strains of the *M. tuberculosis* complex with IS986 as a genetic marker was carried out and showed that strains of *M. tuberculosis* have higher copy numbers of IS986, in agreement with other reports presented in Table 6.1 (Chapter 6). 11 of the 12 the strains of *M. bovis* tested had a single copy of IS986 which was found in a 2Kb *PvuII* restriction fragment. This latter finding is in agreement with many other reports (van Soolingen *et al.*, 1992; Cousins *et al.*, 1993; Skuce *et al.*, 1994; van Soolingen *et al.*, 1994a). In a mass DNA typing study (van Soolingen *et al.*, 1994), a total of 153 *M. bovis* from animals and man from various parts of the world were typed and 82 (53.6%) strains found to contain a single copy of IS986. Moreover, in 56 of them, IS986 was found on a 1.9Kb *PvuII* restriction fragment. The majority of *M. bovis* strains with more than one copy of IS986 had only up to six copies; however, 8 strains of *M. bovis* (mainly from zoo animals) possessed 20 copies of IS986. A thorough review of many other studies involved in typing *M. bovis* on a larger scale than that of the present study is presented in Chapter 6.

Regarding the typing of *M. tuberculosis*, eight of nine strains of *M. tuberculosis* from Tanzania had 10 copies of the IS986 and the same pattern of bands. The similarity of banding pattern among these strains has highlighted the close clonal relationship of *M. tuberculosis* affecting people in Tanzania. Similar observations have been recorded in Malawi and Kenya (Godfrey-Faussett and Stoker 1992) and in Central African states (van Soolingen *et al.*, 1991). Further comparison of DNA fingerprints of strains of *M. tuberculosis* from Tanzania and those reported elsewhere is discussed in Chapter 6.

Plikaytis *et al.* (1992) reported on discrimination of strains of *M. bovis* from *M. tuberculosis* on the basis of DNA fingerprints generated by IS986, which relied on high copy number in *M. tuberculosis* and low copy number in *M. bovis*. The results of the current study concurs with this notion, but the finding of strains of *M. bovis* with multiple copies of IS986 (Skuce *et al.*, 1994; van Soolingen *et al.*, 1994; Szewzky *et al.*, 1995) would make differentiation of *M. bovis* and *M. tuberculosis* on the basis of banding pattern unsuitable in some circumstances.

It, therefore, can be concluded that in general DNA typing and detection procedures provide better tools to detect and type strains of the *M. tuberculosis* complex than traditional techniques. However, in view of the report by van Soolingen *et al.* (1992) and the findings of the present study it can be confirmed that while the standard DNA fingerprinting (IS6110 RFLP typing - van Embden *et al.*, 1993) is appropriate for typing of strains *M. tuberculosis*, it is however, not suitable to type strains of *M. bovis*. The other drawback of RFLP typing, using the Southern blotting procedure is that it requires about 2 micrograms of unsheared DNA for digestion. To obtain this amount requires lengthy culture work.

Regarding PCRs, the present study has found that none of the four protocols was able to detect *M. bovis* specifically, although *mtp40* appear to be specific for *M. tuberculosis* and the rest of the protocols were *M. tuberculosis* complex specific.

It, therefore, was clear that better typing and detection techniques were required in order to conduct a thorough molecular epidemiology of *M. bovis* in Tanzania. There have been reports of the application of simpler PCR based techniques such as random amplification of polymorphic DNA (RAPD) or arbitrarily primed PCR (AP-PCR) for typing a number of species of different microbes with success (Mathews 1993). It was therefore proposed to undertake an investigation on development of simplified techniques for identification and typing of strains of *M. bovis*.

CHAPTER 5
DEVELOPMENT OF MOLECULAR BIOLOGY
TECHNIQUES FOR TYPING AND DETECTION
OF *M. BOVIS*.

CHAPTER 5 DEVELOPMENT OF MOLECULAR BIOLOGY TECHNIQUES FOR TYPING AND DETECTION OF *M. BOVIS*.

5.1. INTRODUCTION

The evaluation of molecular methods currently used in the identification and typing of the *M. tuberculosis* complex fails short of identifying *M. bovis* as a separate species (Chapter 4). This has implication in studies of epidemiology of the disease not only in cattle but more importantly in human tuberculosis due to *M. bovis* infection. Hence attempts are necessitated to develop molecular techniques to identify and type *M. bovis* specifically.

5.1.1. DEVELOPMENT OF SIMPLIFIED MOLECULAR TYPING TECHNIQUE

The commonly used molecular techniques for typing of the *M. tuberculosis* complex include; restriction enzyme analysis (REA), restriction fragment length polymorphism (RFLP) using specific probes (Table 1.5a). The other less famous technique is the pulsed field gel electrophoresis (PFGE). These techniques have received wider application in studies of outbreaks of human tuberculosis, they have successfully established the clonal relationship among strains with a common focal point (Table 6.1& 6.2). The limitation to the use of REA, RFLP and PFGE is that they all require a high quality and quantity of DNA from cultured bacteria, which requires at least four weeks in addition to the first six weeks for primary culture. These delays are compounded by the laborious and cumbersome nature of these techniques.

Recently, attempts have been made to utilise PCR-based techniques to generate fingerprints of strains of mycobacteria, as well as other pathogenic organisms. These techniques (Table 5.1) are potentially advantageous in investigations of epidemiology

of the *M. tuberculosis* complex infection in various situations in similar manner to studies of other micro-organisms (Matthew 1993).

Table 5.1. The PCR typing techniques for the *M. tuberculosis* complex

Technique	Target DNA elements	Species specificity	Reference
Arbitrary Primed PCR	whole genome	<i>M. tuberculosis</i>	Palittapongarnpim <i>et al.</i> 1993a Linton <i>et al.</i> ; 1994
Amplityping	MTPR/IS6110	<i>M. tuberculosis</i>	Plikaytis <i>et al.</i> 1993
Out-PCR	IS6110	<i>M. tuberculosis</i>	Ross and Dwyer 1993
Ligation mediated PCR	IS6110	<i>M. tuberculosis</i>	Palittapongarnpim <i>et al.</i> 1993b
Mixed-Linker PCR	IS6110	<i>M. tuberculosis</i>	Haas <i>et al.</i> 1993

The advantages of the above PCR based techniques over REA, RFLP and PFGE arise not only from their simplicity but also on their ability to type *M. tuberculosis* directly from clinical materials such as sputum (Plikaytis *et al.*, 1993).

With the exception of AP-PRC, the success of the above techniques to differentiate strains of *M. tuberculosis* depend on the presence of IS6110 (IS986) in multiple copies. *M. bovis* on the other hand has either a single or low copy number of IS986 and therefore these same techniques have limited use for typing strains of *M. bovis*, as was discussed in Chapter 4 and by van Soolingen *et al.* (1992). Also, these techniques are not suitable for typing *M. tuberculosis* strains that lack IS986 or with low copy numbers of IS986. Furthermore, some of the techniques, such as those described by Haas *et al.* (1993) and Plikaytis *et al.* (1993), are still cumbersome to perform.

5.1.2. AN ATTEMPT TO DEVELOP A *M. BOVIS* SPECIFIC DNA PROBE.

All organisms, no matter how simple or complex, contain some unique sequences of DNA or RNA within their genomes that distinguish them from all other organisms. It is therefore important that these sequences are identified, isolated and tested for their specificity in hybridization experiments.

According to Tenover (1988), there are several methods which have been widely used to clone specific DNA probes, these include cloning of; virulence genes, random chromosomal fragments, ribosomal RNA, viral DNA or RNA, repetitive sequence from chromosomal DNA, etc.

Approaches to cloning of specific DNA sequences from genus mycobacteria have included, firstly, cloning of the genes responsible for the phenotypic expression of various antigens. These approaches led to specific PCRs for the *M. tuberculosis* complex (Tables 1.5a&b) and other complexes of the genus *Mycobacterium*. As far as *M. bovis* is concerned, a PCR for MPB70, an 18kD major secretory antigen of *M. bovis* (Radford *et al.*, 1988), was originally thought to be specific for the species (Cousin and Wilton 1991). However, the results of Chapter 4 found this PCR to be specific for the *M. tuberculosis* complex.

The second approach used in cloning of mycobacteria specific DNA elements has been the screening of DNA libraries for specific DNA sequences. The DNA elements so far found to be highly conserved and specific for many organisms include genes encoding ribosomal RNA (rRNA) (Fox *et al.*, 1980). rRNA encompasses a 16S rRNA molecule which is functionally constrained, and its' rare changes in certain positions are regarded to be specific to the group or species in which they occur (Dams *et al.*, 1988). Rogall *et al.* (1990), utilised this knowledge and were able to PCR sequence 16SrRNA subunits within a wide range of *Mycobacterium* species and found that the sub-units of 16SrRNA which are found in *Mycobacterium* spp. are 100% homologous thus limiting the ability of this probe to differentiate closely related groups such as the *M. tuberculosis* complex. The 16SrRNA PCR has mainly been used to detect the presence of species of *Mycobacterium* in a culture and in clinical specimens (Boddinghaus *et al.*, 1990). Furthermore, a number Gen-probe preparations also have relied on hybridization of 16SrRNA (Ford *et al.*, 1993).

Subsequent to the findings of Rogall *et al.* (1990), and in a quest to find the *M. tuberculosis* complex specific probes, Zainuddin and Dale (1989) described the isolation and cloning of a fragment of *M. tuberculosis*, approximately 5Kb in size, which contained a repetitive element specific to the *M. tuberculosis* complex organisms. The fragment was found to hybridise to multiple restriction fragments of

different isolates of *M. tuberculosis*, *M. bovis*, *M. bovis* BCG but not in other mycobacteria (Zainuddin and Dale 1989). Probes derived from the 5Kb DNA fragment; IS986 (McAdam *et al.*, 1990), IS6110 (Thierry *et al.*, 1990) and IS987 (Hermans *et al.*, 1991) were found to be highly specific and PCRs designed to amplify these elements have been the key to success in molecular identification of strains of the *M. tuberculosis* complex. The other repetitive DNA element, IS1081, was cloned from *M. bovis* by Collins and Stephen (1991). This element, cloned in a similar manner to IS986 and its variants, has also been found to be specific for the *M. tuberculosis* complex.

In addition to cloning of these mobile elements, a similar approach has resulted in the cloning of other DNA probes currently in use to detect and type strains of the *M. tuberculosis* complex. These include; pMTb4 cloned from *M. tuberculosis*, P36 cloned from *M. tuberculosis* (de Wit *et al.*, 1990), pTBN12 cloned from *M. tuberculosis* (Ross *et al.*, 1992), pTB233 cloned from *M. bovis* (Doran *et al.*, 1993) and pMBA2 cloned recently from *M. bovis* (Bigi *et al.*, 1995). None of the above probes has been found to be specific for *M. bovis*. However, pTBN12, pTB233 and pMBA2 had the ability to detect a (RFLP) generate polymorphism in *M. bovis* that was not detected when the standard IS6110 was used as a probe (Ross *et al.*, 1992; Doran *et al.*, 1993; Bigi *et al.*, 1995).

5.1.3. DEVELOPMENT OF MULTIPLEX PCR ABLE TO DISTINGUISH SPECIES OF THE *M. TUBERCULOSIS* COMPLEX.

As discussed in Chapter 4, polymerase chain reaction, can specifically discriminate the taxon of the *M. tuberculosis* complex from other mycobacteria, but, the existing protocols are unable to discriminate species belonging to the group except *M. tuberculosis* by the *mtp40* PCR. Therefore, as an interim step towards the development of a *M. bovis* specific PCR, which was the major objective of the Bovine Tuberculosis Project at Moredun Research Institute, Edinburgh, U.K., a multiplex PCR capable of distinguishing *M. tuberculosis* from other members of the *M. tuberculosis* complex was developed (Sinclair *et al.*, 1995). The multiplex PCR

was designed to amplify the *M. tuberculosis* complex specific element, IS986 and the *M. tuberculosis* specific element, *mtp40*. This PCR was able to differentiate the closely related members of the tuberculosis complex i.e. *M. tuberculosis* from *M. bovis*. The PCR amplified two DNA fragment from *M. tuberculosis* and a single fragment from *M. bovis*. This procedure has an advantage over the conventional PCR techniques by being able to differentiate mycobacteria of the tuberculosis complex in a single-step PCR as opposed to a nested and hemi-nested PCR, which are time consuming as they require two or three rounds of PCR amplification. The full description of the developed PCR is presented in Appendix V.

5.1.4. AIMS OF THE STUDY

The aim of this exercise was firstly, to develop a simplified PCR protocol which will allow typing of *M. bovis* using PCR by amplifying DNA sequences in between DNA markers specific to the tuberculosis complex, namely IS986, IS1081, MPB70 and *mtp40*. The basis for this amplification is arising from the fact that the mobile genetic elements (IS986 and IS1081) are found at different positions within the genome in unrelated strains, also that the position of single gene elements (MPB70 and *mtp40*) in relation to mobile elements is also variable in unrelated strains and similar in related strains. The PCR typing to be developed is hereby termed the Intersegment PCR.

An attempt also was made to identify an *M. bovis* - specific probe by cloning DNA fragments generated by random amplification of polymorphic DNA (RAPD). The fragments were amplified only in *M. bovis* and absent in other mycobacteria including the other members of the *M. tuberculosis* complex. DNA fragments generated at random may sometimes yield a portion of a specific gene or may be containing a sequence unique to that organism that can be used to develop a specific probe (Tenover 1988). For example, a probe for heat-labile (LT) or heat-stable (ST) enterotoxins of *E. coli* (Moseley *et al.*, 1982), also a DNA probe for detecting *Salmonella* spp. in food samples (Fitts 1985) were developed using this approach (Tenover 1988).

5.20. MATERIALS AND METHODS

5.20.1. INTERSEGMENT PCR

In an attempt to develop a simplified PCR based DNA fingerprinting techniques, a number of PCRs were designed to amplify the regions between transposable DNA elements (IS986 and IS1081) and between those elements and single genes (*mtp40*; MPB70). This procedure was termed intersegment PCR. In intersegment PCR, primers were designed to anneal at the opposite ends of the DNA element, priming outwards from the DNA element and amplifying the DNA in between adjacent similar or different DNA elements as shown in Figures 5.1.&5.2. Primers used in this experiment are indicated in Table 5.2.

A unified master mix was used for all experiments. This was composed of 50mM NaCl, 2.0mM MgCl₂, 10mM Tris-HCl (pH8.3), 0.2mM dNTP's, 0.01% gelatin, 0.5U of *Taq* DNA polymerase and 500ng of each of the primers. 50ng of denatured template DNA from strains of the *M. tuberculosis* complex (MT23, MB10, MB14, MB12 and MT9) was added to the master mix. Primers were designed to have a melting temperature greater than 65°C. Samples were denatured at 94°C for 1 minute, annealed at 65°C for 1 minute and extended at 72°C for 2 minutes.

Seven of the 15 possible permutations were tried to assess this technique in the typing of strains of *M. bovis* and *M. tuberculosis* by exploiting the presence of mobile DNA elements positioned at different locations within the genome (Figure 5.1). An example of how the PCR will be able to type strains is presented in Figure 5.2.

Table 5.2 Primer sequences for intersegment PCR amplifications

Primer ID	Target element	Sequence (5' to 3')	Location (bp)
B976*	IS986 (r)	TGTGCAGATGACTCGACAC	462-443
G4211	IS986 (f)	CCAGCCGCCGGCTGACT	1302-1321
G4210	IS1081 (r)	GCAGCCCGCGCAGCAGATCC	137-118
G4209	MPB70 (r)	TTCGAGGCCGCCACCGCGCA	107-89
G4208	IS1081 (f)	GGACGGCGCTACCTGGGCCT	1190-1209
G4207	<i>mtp40</i> (r)	GGGAACCACCGACGGCGCGT	30-11

f = forward primer r = reverse primer

* Fomoukong *et al.* (1992)

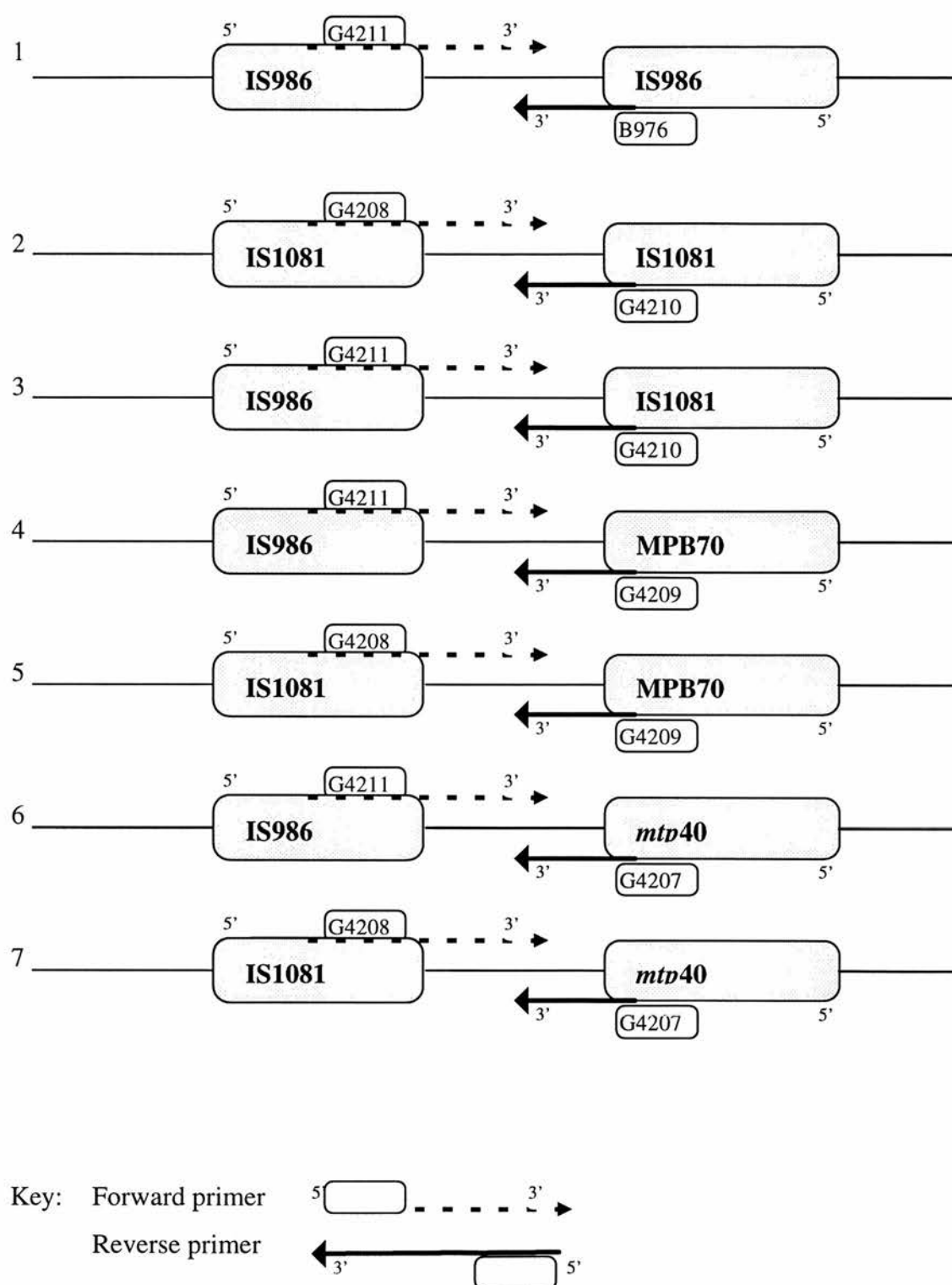
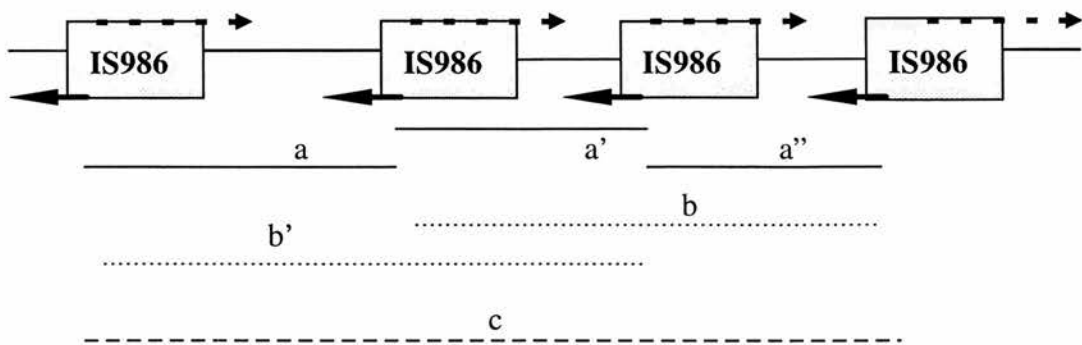


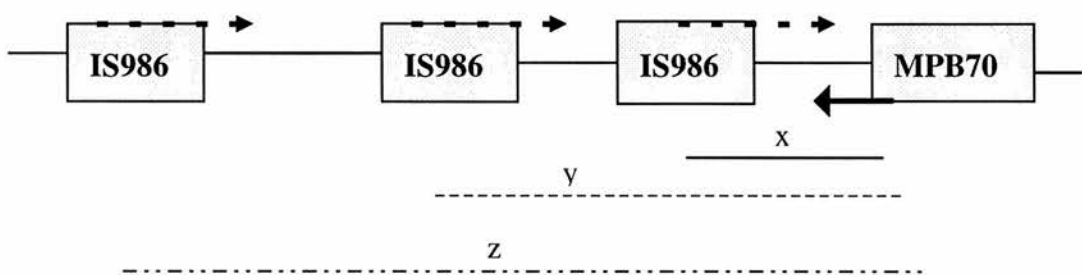
Figure 5.1. The different permutations of intersegment PCR

a) PCR amplification options

i) between repetitive elements (IS986 or IS1081 forward and reverse primers)



ii) between repetitive and single gene elements (e.g. IS986 and MPB70)



b) Intersegment PCR products on gel

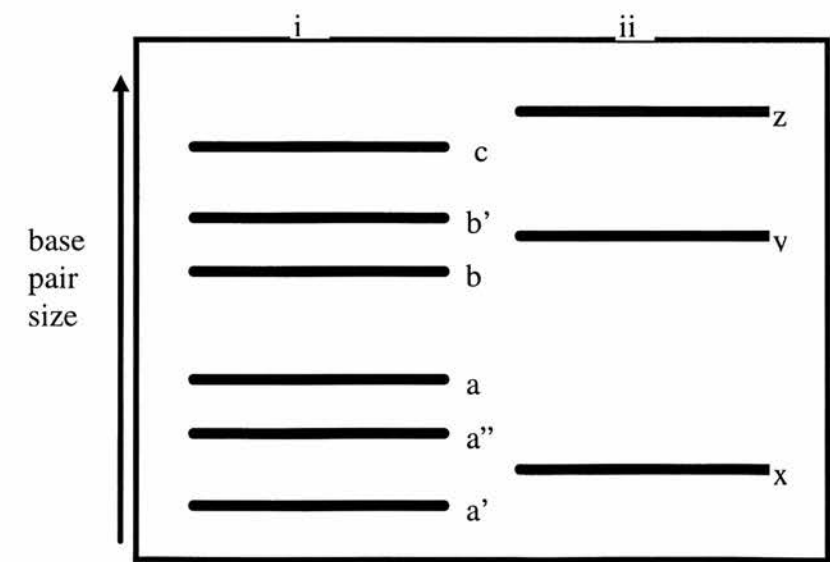


Figure 5.2. Example of DNA fingerprinting by intersegment amplification

5.3. RESULTS

5.3.1. INTERSEGMENT PCR

The different permutations for the intersegment PCR have shown that each of them could produce different fingerprints as shown in Figures 5.3 through 5.9. It appeared with the current sets of primers that the PCR involving IS1081 primers gave multiple and stronger bands but with less polymorphism compared to IS986 primers. The strains included in this experiment were those with known copy numbers of IS986 as follows: MT23, 12 copies; MT9, 2 copies; MB10, MB14 and MB12 one copy. In intersegment PCR using forward (G4211) and reverse (B976) primers for IS986 (Figure 5.3), there was a common band for all the strains at 250bp, and additional bands at 500 and 950bp were present in *M. bovis* (MB14), and *M. tuberculosis* (MT23), and *M. bovis* (MB10) had an additional band at 500bp while in strain *M. bovis* (MB12) and *M. tuberculosis* (MT9) no additional bands were produced. Intersegment PCR which utilised primers amplifying outwards from IS1081 (G4210 and G4208) produced several common bands at 134bp, 200bp, 250bp, and 500bp in all test strains, while polymorphic bands at 480 and 520bp mark were only found in strains MB10 and MT9 (Figure 5.4). Regarding the third option (i.e. primers G4211 and G4210) intersegment PCR for regions between IS986 and IS1081 gave three distinctive bands for all strains at 300bp, 450bp, and 600bp, weak polymorphic bands at 350bp were found also in all strains (Figure 5.5). Intersegment PCR using primers amplifying between IS986 and MPB70 (G4211 and G4209) gave one prominent band at approximately 500bp in all strains. A single band at 240bp was found only in MT23, bands at 250 and 298bp were only found in strains MB10, MB12 and MT9, while numerous bands found between 500bp and 3Kb were produced in strains MB10 and MB14 (Figure 5.6). The intersegment PCR amplifying regions between IS1081 and MPB70 (Figure 5.7) produced a similar number of bands between 240bp and 3.0Kb in strains MT23, MT9 and MB10; however the last two strains had a extra band at 100bp. Strain MB12 produced a band in common with the above-mentioned strains between 240bp and 600bp. Strain MB12 produced a totally different banding

pattern. This produced 3 prominent bands between 50 and 100bp. Intersegment PCR amplifying regions between IS986 and *mtp40* using primers G4211 and G4207 produced one distinctive PCR product at 300bp in all test strains. The weak bands also common to all strains were found at 220bp and 1010bp; there was no polymorphism in this protocol (Figure 5.8). Intersegment PCR amplifying regions between IS1081 and *mtp40* produced similar bands in all strains between 220bp and 600bp, and at 1.6Kb. Polymorphic bands were those found between 600bp and 1.6Kb. Strains MT23, MB14 and MB12 produced a similar band pattern, different to that produced by strains MB10 and MT9 (Figure 5.9).

The results of optimisation experiments in Chapter 4 showed that the number of non-specific PCR products were due to an increased concentration of template DNA. In the current study the concentration of template DNA was doubled (i.e. to 100ng) in order to determine its influence on generation of polymorphic DNA fragments, using primers amplifying regions between IS986 (G4211 and B976) and IS1081 (G4208 and G4210). In comparison with the banding patterns shown in Figures 5.3 and 5.4, the results of intersegment PCR for IS986 and IS1081 using a high concentration of template DNA gave numerous polymorphic bands (i.e. from 75bp to 3Kb) (Figures 5.10a&b). Experiments on reducing the amount of template DNA by diluting DNA 1:10 (i.e. to 5ng) using the same set of strains and same PCR protocol, resulted in amplification of a single band in only a few strains while the majority did not yield any detectable product (data not shown).

In order to test for specificity of the intersegment PCR, primers amplifying regions between IS986 were applied to strains of atypical mycobacteria viz. *M. kansasii*, *M. malmoense*, *M. gordonae*, *M. marinum* and *M. fortuitum* (Appendix IV). The results (Figure 5.11) indicate the presence of PCR products in all of the MOTTs tested. No similar banding pattern was reproduced in any of the MOTTs. Furthermore, none of the banding patterns resembled those found among strains of the *M. tuberculosis* complex tested using similar intersegment PCR conditions (Figure 5.4).

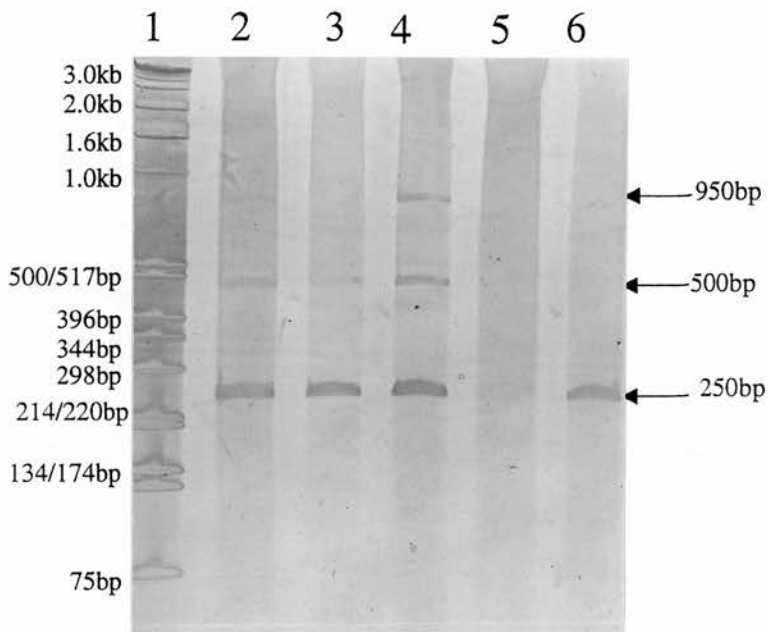


Figure 5.3. IS986 \leftrightarrow IS986 (primers G4211/B976) intersegment PCR patterns observed on silver stained 8% polyacrylamide gel. Lane 1 , 1Kb ladder; Lane 2, *M. tuberculosis* (MT23); Lane 3, *M. bovis* (MB10); Lane 4, *M. bovis* (MB14); Lane 5, *M. bovis* (MB12); Lane 6, *M. tuberculosis* (MT9). Arrows on the right point to to the most prominent polymorphic bands, figures on the left indicate molecular size in base pairs and lane numbers are indicated at the top.

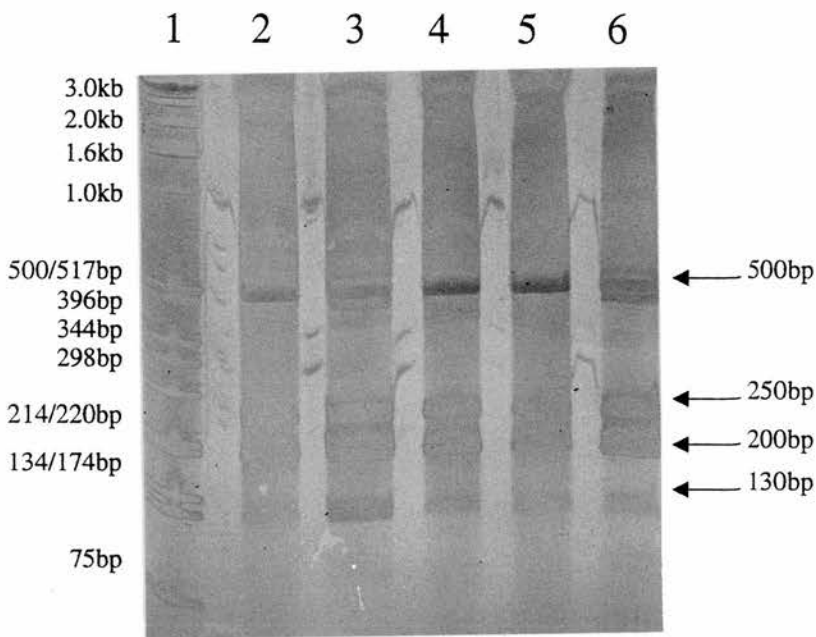


Figure 5.4. IS1081↔IS1081 (primers G4208/G4210) intersegment PCR patterns observed on silver stained 8% polyacrylamide gel. Lane 1 , 1Kb ladder; Lane 2, *M. tuberculosis* (MT23); Lane 3, *M. bovis* (MB10); Lane 4, *M. bovis* (MB14); Lane 5, *M. bovis* (MB12); Lane 6, *M. tuberculosis* (MT9). Arrows on the right point to the most prominent polymorphic bands, figures on the left indicate molecular size in base pairs and lane numbers are indicated at the top.

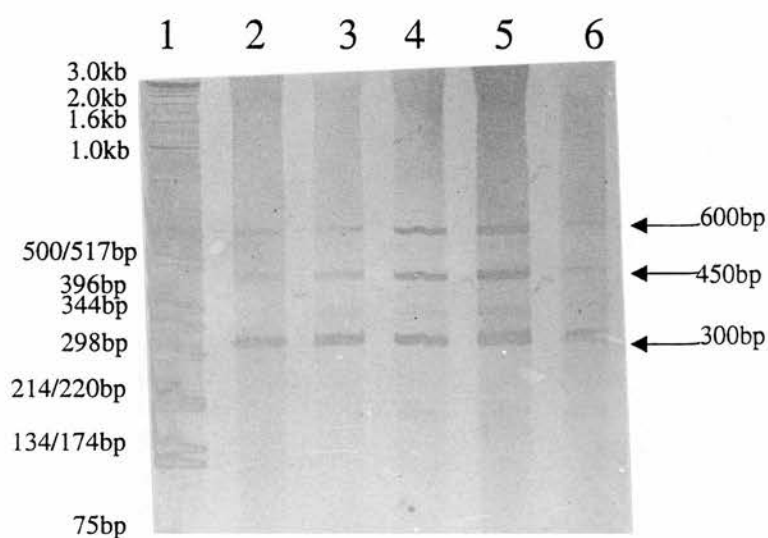


Figure 5.5. IS986↔IS1081 (primers G4211/G4210) intersegment PCR patterns observed on silver stained 8% polyacrylamide gel. Lane 1 , 1Kb ladder; Lane 2, *M. tuberculosis* (MT23); Lane 3, *M. bovis* (MB10); Lane 4, *M. bovis* (MB14); Lane 5, *M. bovis* (MB12); Lane 6, *M. tuberculosis* (MT9). Arrows on the right point to the most prominent polymorphic bands, figures on the left indicate molecular size in base pairs and lane numbers are indicated at the top.

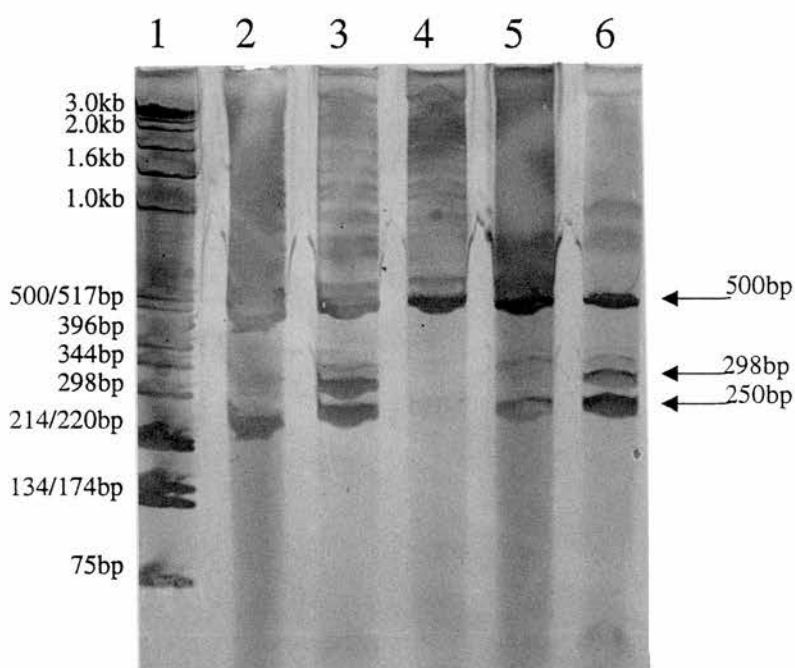


Figure 5.6. IS986↔MPB70 (primers G4211/G4209) intersegment PCR patterns observed on silver stained 8% polyacrylamide gel. Lane 1 , 1Kb ladder; Lane 2, *M. tuberculosis* (MT23); Lane 3, *M. bovis* (MB10); Lane 4, *M. bovis* (MB14); Lane 5, *M. bovis* (MB12); Lane 6, *M. tuberculosis* (MT9). Arrows on the right point to the most prominent polymorphic bands, figures on the left indicate molecular size in base pairs and lane numbers are indicated at the top.

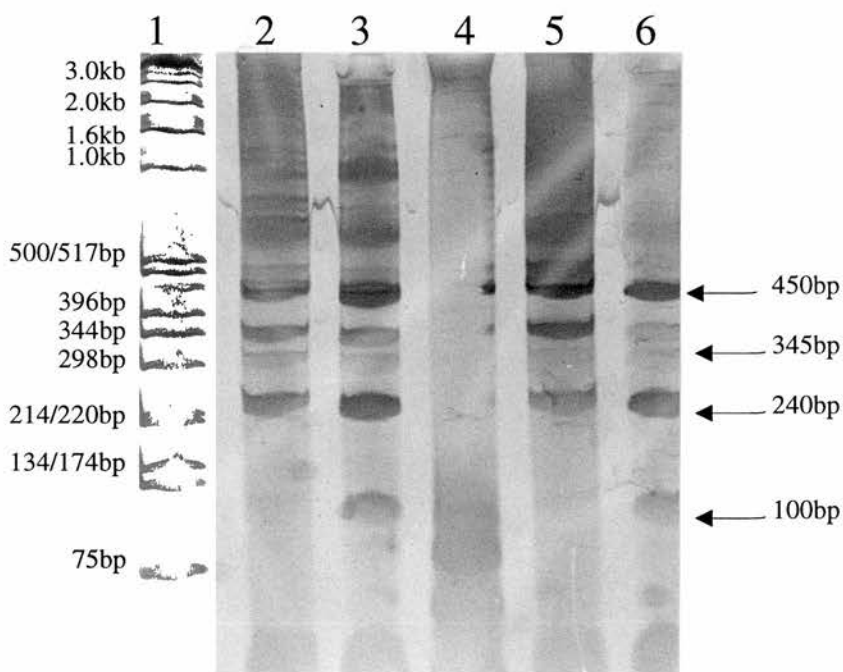


Figure 5.7. IS1081↔MPB70 (primers G4211/B976) intersegment PCR patterns observed on silver stained 8% polyacrylamide gel. Lane 1 , 1Kb ladder; Lane 2, *M. tuberculosis* (MT23); Lane 3, *M. bovis* (MB10); Lane 4, *M. bovis* (MB14); Lane 5, *M. bovis* (MB12); Lane 6, *M. tuberculosis* (MT9). Arrows on the right point to the most prominent polymorphic bands, figures on the left indicate molecular size in base pairs and lane numbers are indicated at the top.

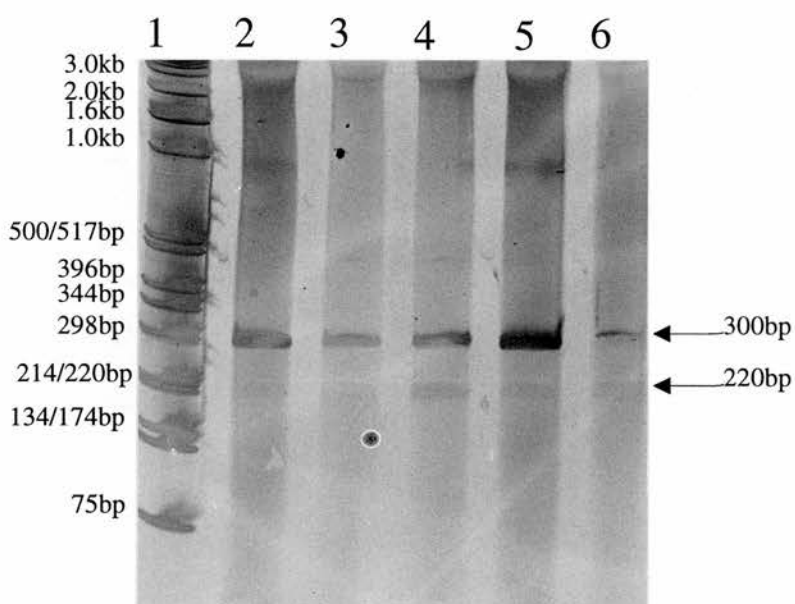


Figure 5.8. IS986 \leftrightarrow mtp40 (primers G4211/G4207) intersegment PCR patterns observed on silver stained 8% polyacrylamide gel. Lane 1 , 1Kb ladder; Lane 2, *M. tuberculosis* (MT23); Lane 3, *M. bovis* (MB10); Lane 4, *M. bovis* (MB14); Lane 5, *M. bovis* (MB12); Lane 6, *M. tuberculosis* (MT9). Arrows on the right point to the most prominent polymorphic bands, figures on the left indicate molecular size in base pairs and lane numbers are indicated at the top.

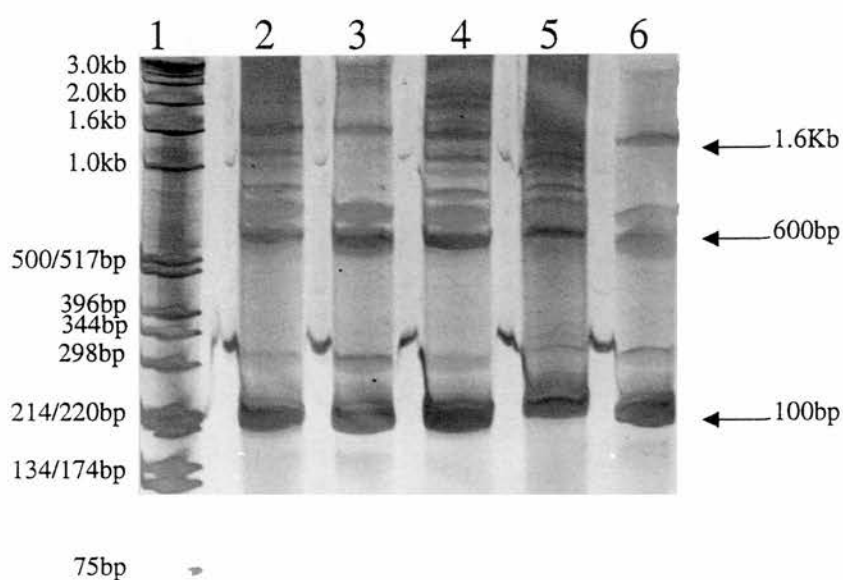


Figure 5.9. IS1081↔*mtp*40 (primers G4208/G4207) intersegment PCR patterns observed on silver stained 8% polyacrylamide gel. Lane 1 , 1Kb ladder; Lane 2, *M. tuberculosis* (MT23); Lane 3, *M. bovis* (MB10); Lane 4, *M. bovis* (MB14); Lane 5, *M. bovis* (MB12); Lane 6, *M. tuberculosis* (MT9). Arrows on the right point to the most prominent polymorphic bands, figures on the left indicate molecular size in base pairs and lane numbers are indicated at the top.

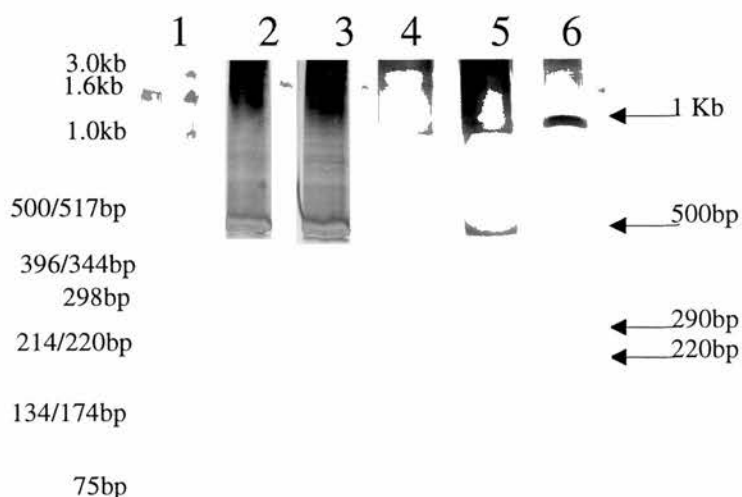
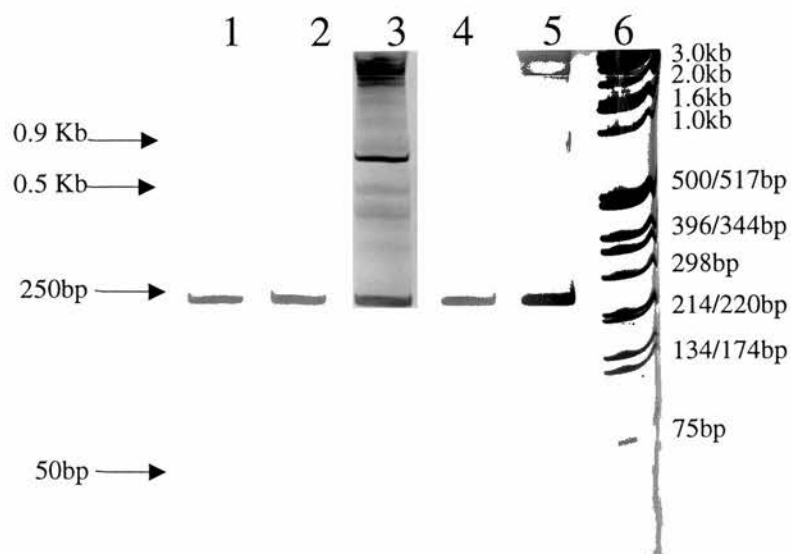


Figure 5.10a&b. Sensitivity of intersegment PCR on a high concentration of template DNA (100ng). **a)** IS986 \leftrightarrow IS986 (primers G4211/B976) intersegment PCR patterns observed on silver stained 8% polyacrylamide gel. Lane 1 , *M. tuberculosis* (MT9); Lane 2, *M. tuberculosis* (MT23); Lane 3, *M. bovis* (MB10); Lane 4, *M. bovis* (MB14); Lane 5, *M. bovis* (MB12); Lane 6, 1Kb ladder. The figures on the left indicate molecular size in base pairs. **b)** IS1081 \leftrightarrow IS1081 (primers G4211/B976) intersegment PCR patterns observed on silver stained 8% polyacrylamide gel. Lane 1 , 1Kb ladder; Lane 2, *M. tuberculosis* (MT23); Lane 3, *M. bovis* (MB10); Lane 4, *M. bovis* (MB14); Lane 5, *M. bovis* (MB12); Lane 6, *M. tuberculosis* (MT9). Arrows point to the most prominent polymorphic bands. Figures on the right (a)/left (b) indicate molecular size in base pairs and lane numbers are indicated at the top.

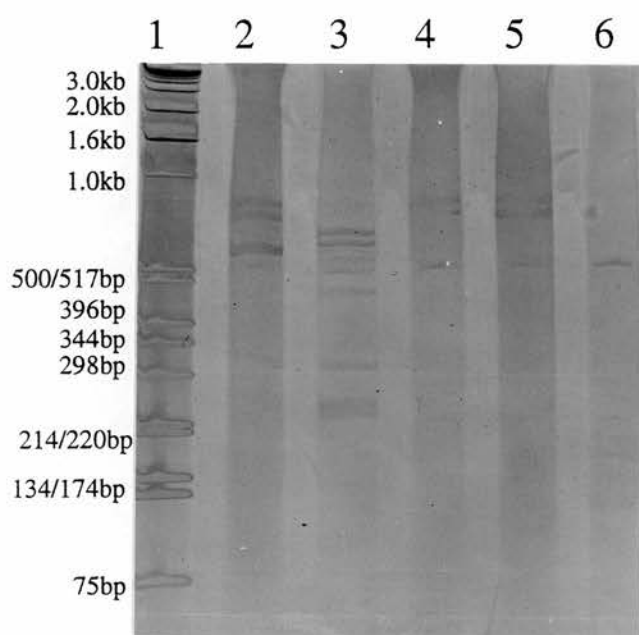


Figure 5.11. IS986↔IS986 (primers G4211/B976) intersegment PCR patterns produced by strains of atypical mycobacteria observed on silver stained 8% polyacrylamide gel. Lane 1 , 1Kb ladder; Lane 2, *M. kansasii* (MOTT3); Lane 3, *M. goodii* (MOTT1); Lane 4, *M. marinum* (MOTT2); Lane 5, *M. malmoense* (MOTT4); Lane 6, *M. fortuitum* (MOTT5). Figures on the left indicate molecular size in base pairs and lane numbers are indicated at the top.

5.3.2. DEVELOPMENT OF *M. BOVIS* SPECIFIC PROBE

5.3.2.1. Random Amplifying Polymorphic DNA (RAPD) PCR

The RAPD PCR has been used successfully to type of strains of various micro-organisms (Matthew 1993). The molecular basis of this procedure is that short oligonucleotides have the ability to prime non-specifically within the genome and produce, at random, numerous PCR products. The procedure may provide both clues to genomic similarity among strains of the same species, and also genetic differences between closely related species such as members of the *M. tuberculosis* complex. In an attempt to obtain a *M. bovis* specific probe, RAPD was applied to *M. bovis* strains MB33, MB11 and MB1 using a single 10 oligonucleotide primer, G6007 (GGACTGGCAT). The PCR master mix was composed of: 25mM dNTPs, 1 X PCR reaction buffer, 20pmol/μl primer and 1U *Taq* DNA polymerase. Fully denatured 25-50ng of template DNA was added to the master mix. The final volume for this PCR was 100μl. In order to minimise the number of non-specific amplification products, the annealing temperature was set at 40°C (higher than the melting temperature of the primer 32°C). The three step PCR cycle was carried out as follows: denaturation at 94°C for 30 seconds, annealing at 40°C for 30 seconds and extension at 72°C for one minute, a total of 40 cycles were performed in this experiment.

The visualisation of the product was carried out on 1% low melting point agarose gels so that appropriate DNA fragments could be excised and recovered from the gel by the method described in 2.2.2.4.4. (Chapter 2).

Two fragments, termed *f2b* and *f3b* were observed in *M. bovis* and not in *M. tuberculosis* (Figure 5.12) and identified as containing potential *M. bovis* specific sequences.

5.3.2.2. Cloning of *f2b* and *f3b* RAPD PCR fragments

These two RAPD products were therefore cloned by cutting out the gel fragments, extracting each fragment (see above) and cloning into pGEM[®]-T vector.



Figure 5.12. Agarose gel showing amplimers of RAPD PCR for strains of *M. bovis* and *M. tuberculosis* indicating the fragments *f2b* and *f3b*. Lane 1 , 1Kb ladder; Lanes 2, 3 and 4, 10, 50 and 100ng of DNA of *M. bovis* (MB31); Lanes 5, 6 and 7, 10, 50 and 100ng of DNA of *M. bovis* (MB33); Lanes 8, 9 and 10, 10, 50 and 100ng of DNA of *M. bovis* (MB29); Lanes 11, 12 and 13, 10, 50 and 100ng of DNA of *M. bovis* (MB11); Lane 14, *M. tuberculosis* (MT8); Lane 15, *M. tuberculosis* (MT43); Lane 16, *M. tuberculosis* (MT35). Arrows on the left point to the cloned fragments, figures on the left indicate molecular size in base pairs and lane numbers are indicated at the top.

Successful cloning was indicated by growth of white colonies about a millimetre in diameter, while transformed cells without a cloned fragment maintained their β -galactosidase activity, and hence produced blue pin point colonies. Further confirmation of cloning was provided by cleaving the fragments of 200bp and 350bp for *f3b* and *f2b*, respectively, from the pGEM[®]-T vector using endonucleases *KspI* (CCGC↓GG) and *SpeI* (A↓CTAGT) whose restriction sites are 7bp on the left and 3bp on the right respectively from the cloning site of the pGEM[®]-T vector respectively (data not shown).

5.3.2.3. Sequencing of cloned *f2b* and *f3b* DNA fragments

The sequences for primers used to complete the sequencing of the fragments are indicated in Table 5.3. The reverse primer for pGEM[®]-T was found not to be suitable as its annealing site was 110bp away from the cloning site. Therefore, it was decided to design a primer (T4442) to anneal at 18bp from the cloning site of pGEM[®]-T.

Table 5.3. Primers used to sequence cloned *f2b* and *f3b* RAPD PCR fragments

Primer ID	DNA sequence	priming	direction	position*
pGEM-T _f	GTAAAACGACGGCCAAGT	pGEM [®] -T**	forward	2964-2980
pGME-T _r	CAGGAAACAGCTATTGAC	pGEM [®] -T	reverse	177-161
T4442	CATATGGTTCGACCTGCA	pGEM [®] -T	reverse	86 - 69
T4444	AACTTTGGAGAGAGACA	<i>f2b</i>	forward	96 - 112
T5985	CACAGCTGAAGCTCACG	<i>f2b</i>	reverse	188 - 172
T4443	CCACGAATGATGATGTT	<i>f3b</i>	forward	125 - 141

* Primer position in 5'- 3' direction

** pGEM[®]-T Accession number X65308

5.3.2.4.. Sequence analysis

Using the University of Wisconsin Genetics Computer Group (UWGCG) programmes (Devereux *et al.*, 1984) available at SERC Daresbury Laboratory SEQNET Computing Facilities, the nucleic acid sequences obtained by the dideoxynucleotide chain termination sequencing and the deduced amino acid

sequences of *f2b* (Figure 5.13) and *f3b* (Figure 5.14a) were analysed and revealed that *f2b*, had 41.5% GC content, contained no open reading frames on either strand, and had 5 to 7 stop codons in all six reading frames. *f3b* had 57.6% GC content and have 3 open reading frames. The first reading frame was on the forward strand and the second and third reading frames on the reverse strand; one to three stop codons were found in the remaining reading frames.

The two fragments were submitted to EMBL Nucleotide Sequence Database and were given accession numbers; Z82980 for *f3b* and Z82981 for *f2b*.

The EMBL database was searched for sequences homologous to *f2b* and *f3b* using the FASTA (UWGCG) programme and in order to ensure that the fragments were properly sequenced a comparison also was made between the DNA sequences of *f2b* and *f3b* and that of the pGEM[®]-T vector. This was to ensure that insert DNA i.e. RAPD product had been sequenced and not the vector. The OWL database (Bleasby and Wootton 1990) was searched for homologous proteins using MPSRCH (Collins and Sturrock 1994). No homologous peptide sequence was found using this search. A search on the Internet nucleotide and peptide sequence database revealed homology between the *f3b* sequence and that of a *M. tuberculosis* cosmid (Accession number Z80343). 75% of the amino acid sequence of this cosmid resembled the peptide sequence of *rfbE* gene product of *Yersinia enterocolitica*. Moreover, the homology between the cosmid and *rfbE* gene in the region similar to *f3b* was only 45.3% (Figure 5.14b).

Comparison was also made using UWGCG software of the DNA sequence of *f2b* and *f3b* on both forward and reverse strands with that of the pGEM[®]-T vector to rule out the possibility that the sequences obtained might have been those of the pGEM[®]-T vector. No significant homology was observed for either *f2b* and *f3b*. For *f2b*, stretches of between 23 to 25 bases showed 72% homology while for *f3b*, 32 to 37 bases had a similarity of 78%, which indicates in both cases that they did not derive from the pGEM[®]-T vector.

```

a      W T G M A S N * F E S S F S L T L R N Q
b      G L A W Q V T D L K A L F H * L Y E I S
c      D W H G K * L I * K L F F T D F T K S V
TGGACTGGCATGGCAAGTAACTGATTTGAAAGCTCTTTTTCAGTACTTTACGAAATCAG
1  -----+-----+-----+-----+-----+-----+60
ACCTGACCGTACCGTTCATTGACTAACTTTTCGAGAAAAAGTGACTGAAATGCTTTAGTC
d      S Q C P L Y S I Q F S K K V S K V F D
e      V P M A L L Q N S L E K E S V K R F *
f      P S A H C T V S K F A R K * Q S * S I L

a      * R C H R L W C F R A V T L E R D K T *
b      E D A T D Y G A S E Q * L W R E T K H E
c      K M P Q I M V L Q S S N F G E R Q N M S
TGAAGATGCCACAGATTATGGTGCTTCAGAGCAGTAACTTTGGAGAGAGACAAAACATGA
61 -----+-----+-----+-----+-----+-----+120
ACTTCTACGGTGTCTAATACCACGAAGTCTCGTCATTGAAACCTCTCTCTGTTTGTACT
d      T F I G C I I T S * L L L K P S L C F M
e      H L H W L N H H K L A T V K S L S L V H
f      S S A V S * P A E S C Y S Q L S V F C S

a      V C I F W P L P R H V K K V L H L R E L
b      F V S S G H F L G T * R R C C T S V S F
c      L Y L L A T S * A R E E G A A P P * A S
GTTTGTATCTTCTGGCCACTTCCTAGGCACGTGAAGAAGGTGCTGCACCTCCGTGAGCTT
121 -----+-----+-----+-----+-----+-----+180
CAAACATAGAAGACCGGTGAAGGATCCGTGCACTTCTTCCACGACGTGGAGGCACTCGAA
d      L K Y R R A V E * A R S S P A A G G H A
e      T Q I K Q G S G L C T F F T S C R R S S
f      N T D E P W K R P V H L L H Q V E T L K

a      Q L C H L * I G S L K C N G I Q * D Q I
b      S C V T Y K L G V * S V M E F N E I K *
c      A V S P I N W E F K V * W N S M R S N K
CAGCTGTGTACCTATAAATTGGGAGTTTAAAGTGTAATGGAATTCAATGAGATCAAATA
181 -----+-----+-----+-----+-----+-----+240
GTGACACAGTGGATATTTAACCCTCAAATTTACATTACCTTAAGTTACTCTAGTTTAT
d      E A T D G I F Q S N L T Y H F E I L D F
e      * S H * R Y I P L K F H L P I * H S * I
f      L Q T V * L N P T * L T I S N L S I L Y

a      R K V L G H M V * L Q Y S L Q V N S I V
b      E K Y L A T W Y D C N I V C K S I L L L
c      K S T W P H G M T A I * S A S Q F Y C C
AGAAAAGTACTTGGCCACATGGTATGACTGCAATATAGTCTGCAAGTCAATTCTATTGTT
241 -----+-----+-----+-----+-----+-----+300
TCTTTTCATGAACCGGTGTACCATACTGACGTTATATCAGACGTTCAAGTTAAGATAACAA
d      L F L V Q G C P I V A I Y D A L * N * Q
e      L F T S P W M T H S C Y L R C T L E I T
f      S F Y K A V H Y S Q L I T Q L D I R N N

a      A I S V M P V
b      L L V * C Q S
c      Y * C D A S P
GCTATTAGTGTGATGCCAGTCCA
301 -----+-----+-----+-----+-----+323
CGATAATCACACTACGGTCAGGT
d      Q * * H S A L G
e      A I L T I G T W
f      S N T H H W D

```

Figure 5. 13. The nucleotide sequence (ordinary letters) and deduced amino acid sequence (bold capital letters) of *f2b*. **a**, **b** and **c**, are the reading frames on forward DNA strand while **d**, **e** and **f** are the reading frames on reverse DNA strands. * stop codon.

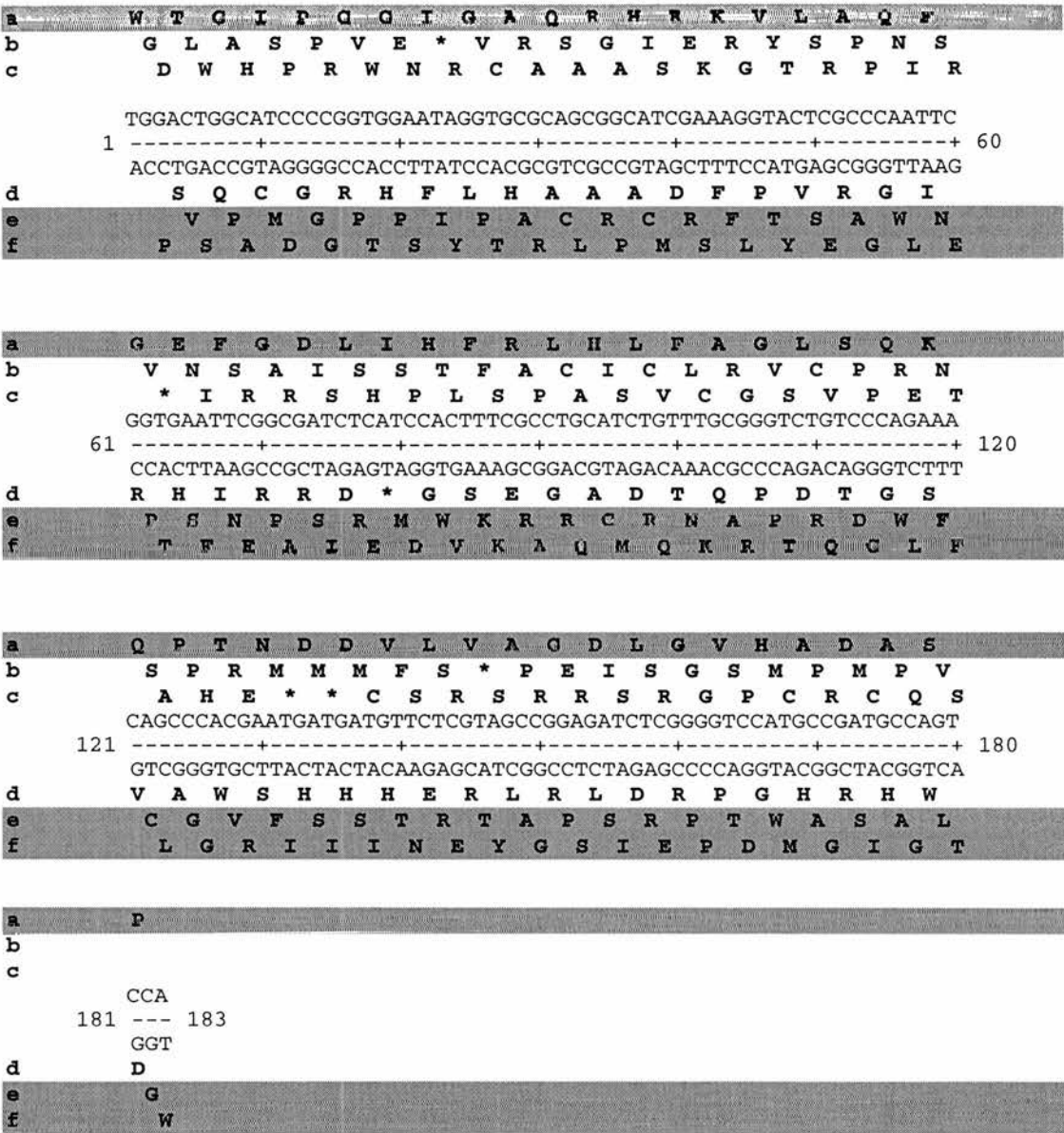



Figure 5. 14a. The nucleotide sequence (ordinary letters) and deduced amino acid sequence (bold capital letters) of *f3b*. **a**, **b** and **c**, are the reading frames on forward DNA strand while **d**, **e** and **f** are the reading frames on reverse DNA strands. * stop codon.  open reading frame.

<i>f3b</i>	G	M	D	P	E	I	S	G	Y	E	N	I	I	R	G	L	F	L	G	Q	T	R	K	
<i>M.tb</i> cosmid	G	M	D	P	E	I	S	G	Y	E	N	I	I	R	G	L	F	L	G	Q	T	R	K	
<i>Y. enterocolitica</i>	G	M	D	P	E	L	S	G	Y	E	N	I	M	R	M	G	L	L	L	G	N	S	V	A
Concesus	*	*	*	*	*	-	*	*	*	*	*	*	-	-	-	*	*	-	*	*	-	-	-	-

<i>f3b</i>	Q	M	Q	A	K	V	D	E	I	A	E	F	T	E	L	G	E	Y	L	S	M	P	L	R
<i>M.tb</i> cosmid	Q	M	Q	A	K	V	D	E	I	A	E	F	T	E	L	G	E	Y	L	S	M	P	L	R
<i>Y. enterocolitica</i>	S	A	K	S	K	I	P	E	I	E	E	F	C	E	L	G	D	F	L	V	L	P	V	R
Concesus	-	-	-	-	*	-	-	*	*	-	*	*	-	*	*	*	-	-	*	-	-	*	-	*

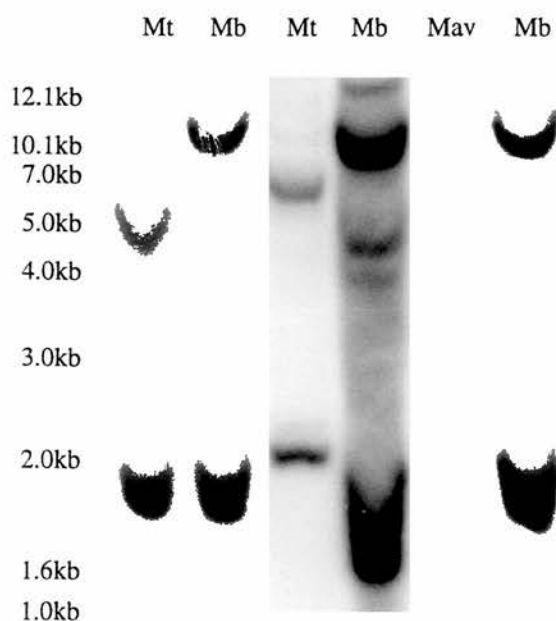
<i>f3b</i>	T	Y	S	T	G
<i>M.tb</i> cosmid	T	Y	S	T	G
<i>Y. enterocolitica</i>	T	Y	S	S	G
Concesus	*	*	*	-	

Figure 5.14b. Peptide sequence match between *f3b* (Accession number Z82980), *M. tuberculosis* cosmid (Accession number Z80343) and *rfbE* gene of *Yersinia enterocolitica* (Accession number Z18920). * consesus peptides - non similar peptides

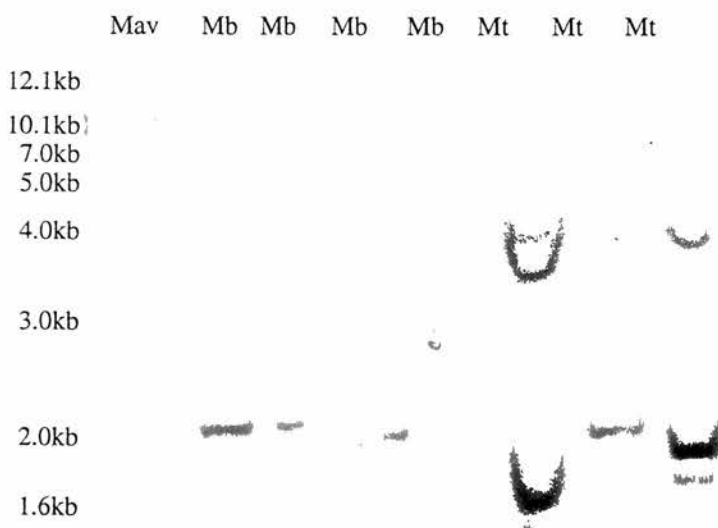
Comparison was also made between the DNA sequence of Primers T4444 and T5985 which were used to complete the sequencing of *f2b* with the DNA sequence of pGEM®-T vector and also in producing the probe for the hybridisation experiment. Although there were matches DNA up to 90% which might amplify a PCR product, these matches occurred far apart within the vector and would generate a band of over 1Kb as opposed to the 93bp generated by the same primers when insert DNA is used.

5.3.2.5. Specificity of the sequenced DNA for *f2b* and *f3b* for *M. bovis*

In order to determine the specificity of the two fragments for *M. bovis*, Southern blot hybridisation experiments were performed in a similar manner to that described in 2.2.2.2. (Chapter 2) using field strains of *M. bovis*, *M. tuberculosis*, *M. terrae*, and *M. avium* (Chapter 3). Three experiments were carried out using the following DNA as probes for each experiment: i) the original *f2b* and *f3b* DNA from RAPD PCR, ii) entire *f2b* and *f3b* fragments cleaved from the pGEM[®]-T vector, and iii) portions of the *f2b* and *f3b* DNA sequences a smaller portion of *f2b* was obtained by further PCR using primers T4444 and T5985 that amplified a 93bp region (position 96-188 on sequence in Figure 5.13) of pGEM[®]-T insert. The fragment of *f3b* was obtained by digesting the clone with *Bgl*III and *Eco*RI to yield a fragment of 99bp (position 64-153 on sequence in Figure 5.14a). These experiments were carried out in three phases. In the first phase the amplified product from the RAPD PCR was used as a probe on a Southern blot containing *M. tuberculosis*, *M. bovis* and atypical mycobacteria. In this exercise, the two fragments hybridised to both *M. tuberculosis* and *M. bovis* DNA giving multiple bands (Figure 5.15a and b). Similar results were obtained when the fragments were excised from the pGEM[®]-T vector (data not shown). When DNA sequences internal to the RAPD PCR primer sites were used as a probe, a single band at approximately 2.0Kb was hybridised using *f3b* sequence (Figure 5.16) while no hybridisation product was detected with DNA from *f2b*. The reasons for the failure in obtaining hybridisation product with *f2b* might be due to problems associated with the preparation of the probe or within the hybridisation protocol itself. These problems were not resolved within the time scale of this experiment.



a) f2b RAPD fragment



b) f3b RAPD fragment

Figure 5.15a&b. Southern blot hybridisation of genomic DNAs of *M. tuberculosis* (Mt), *M. bovis* (Mb) and *M. avium* (Mav) with a) *f2b* and b) *f3b* RAPD PCR products as probes.

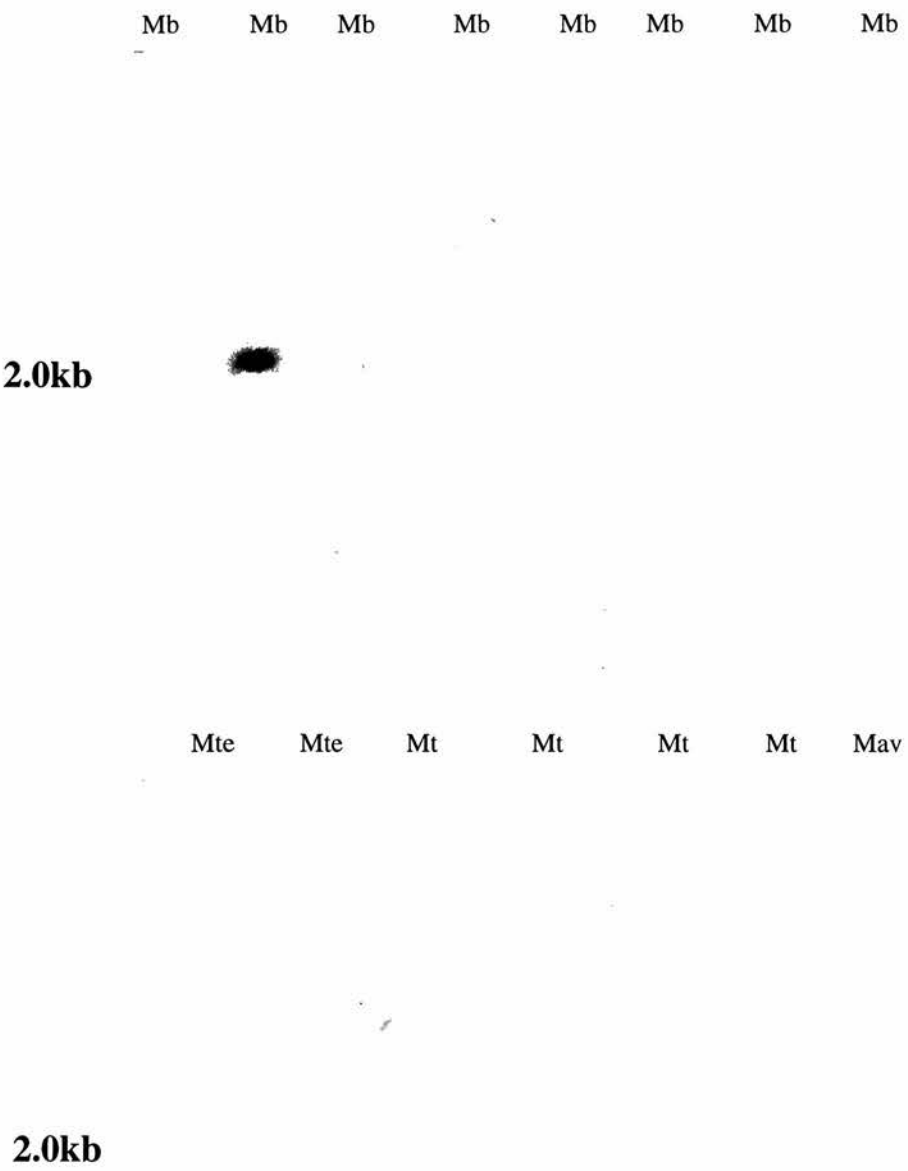


Figure 5.16. Southern blot hybridisation with a DNA probe obtained from *BglIII/EcoRI* digestion of the *f3b* from cloned DNA. Strains analysed; *M. bovis* (Mb), *M. tuberculosis* (Mt), *M. avium* (Mav) and *M. terrae* (Mte)

5.4. DISCUSSION

5.4.1. PCR FINGERPRINTING

The inability of the standard RFLP typing technique (van Embden *et al.*, 1993) to differentiate test strains of *M. bovis* in this study (Chapter 4), as well as the experience of other laboratories (van Soolingen *et al.*, 1992), prompted this study to look for an alternative approach for typing of the *M. bovis* strains. An alternative approach developed in the present study was a PCR based technique which was designed to amplify the regions between specific DNA elements of the *M. tuberculosis* complex. The technical simplicity combined with sensitivity and speed of the PCR was one of the main reasons for conducting PCR typing experiments.

The results of the intersegment PCR typing showed that this technique could type the strains of the *M. tuberculosis* complex, particularly strains of *M. bovis*. The strains of *M. bovis* used in this exercise were initially typed by the standard IS6110 RFLP typing (van Embden *et al.*, 1993). The intersegment PCR using primers derived from specific DNA elements was able to generate multiple bands, thus allowing further strain subtypes to be identified compared to less polymorphic fingerprints generated by the standard IS986 RFLP typing, where the majority of *M. bovis* strains had a single band at 1.9Kb (Figure 4.10 vs Figures 5.3 through 5.9).

The number of bands and polymorphisms generated by intersegment PCR was generally greater when primers were based on IS986 than IS1081. This finding is in agreement with those obtained by van Soolingen *et al.* (1992) and Collins *et al.* (1993). The latter found only four of 171 strains of the *M. tuberculosis* complex to have a different IS1081 RFLP pattern, the rest of the strains had an identical fingerprint pattern. Results of intersegment PCR using primers from IS986 and IS1081, revealed three distinctive bands at approximately 300bp, 500bp and 700bp in all the strains, which could be an indication of either the relative position of one of the IS986 to a number of IS1081 is the same in the majority of strains, or that one of the primers had annealed at three sites which are found at the same distance in relation to IS1081 or IS986 in all the strains of the *M. tuberculosis* complex. Less

polymorphism also was exhibited when a combination of primers from the mobile element IS1081 and single genes, MPB70 and *mtp40*, were used for intersegment PCR (Options 6 and 7), which is in contrast to combinations of primers involving IS986 and either *mtp40* or MPB70. This finding agrees with many others which have found that the use of IS986 as a probe generates a high degree of polymorphism in unrelated strains compared to IS1081 (van Soolingen *et al.*, 1992; van Embden *et al.*, 1992; Collins *et al.*, 1993; van Embden *et al.*, 1993).

In most of PCR based techniques the quantity and integrity of the product is a function of many parameters, such as annealing temperature, concentration of magnesium salt, dNTPs, *Taq* polymerase and template DNA (Wagner, Jr. and Fowler 1993). The results of optimisation of PCR discussed in Chapter 4 implicated template DNA to be the cause of non-specific amplification products. The current study also found that high template DNA concentration was responsible for a number of extra bands on IS986↔IS986 and IS1081↔IS1081 when twice as much DNA (100ng) was used in PCR reaction (Figures 5.11a&b). A reduced DNA concentration resulted in a low number or absence of bands produced by the PCRs (data not shown). This increase in number of products amplified as a result in increased DNA concentration is similar to that observed by Davin-Regli *et al.* (1995) who implicated the difference in RAPD banding pattern to concomitant changes in three different molecular conditions, namely; perfect annealing sites, existence of rare sites, and mismatching events. At high template concentration, the number of strong bands is high. This probably corresponds to either perfect annealing sites, multiple copies of annealing site or best annealing events, while at low concentration the bands observed are due to rare molecular events which might occur during the first cycles of PCR amplification, or amplification as a result of mismatch annealing events due to the rarity of perfect priming sites or inaccessible targets (Davin-Regli *et al.*, 1995).

The results of the intersegment PCR although promising, were put further into question when it was realised that some strains (MB10; MB12; MB14) contain only a single copy of IS986 in their genome (Figure 4.10) but produced multiple bands in a IS986↔IS986 intersegment PCR. This finding implied that one or both of the primers used may have been priming to homologous structures outside the IS986. A

similar experience has been recorded by other workers (Ross and Dwyer 1993; Neimark *et al.*, 1996). These workers sequenced the amplified DNA products amplified and found that some of the fingerprint bands were primed from non-IS986 sequences. These workers implied that this non-specific priming was a result of low annealing temperature and higher magnesium concentration (Ross *et al.*, 1993).

In view of the above discussion, it could easily be concluded that the results of intersegment PCR resembled those of RAPD PCR or AP PCR (Palittapongpim *et al.*, 1993), but there are several significant differences between intersegment PCR and AP PCR: i) the size of primers used in the current study were between 18 and 20 mer as opposed to 10 mers used in most AP PCRs ii) the sequence of primers were those found within the respective DNA elements, while in AP PCR randomly selected DNA is used, iii) annealing temperature was set at high stringency (i.e. not less than 5°C below primer melting temperature), as compared to 25° to 40°C commonly used in AP PCR, and iv) the concentration of magnesium used throughout the experiments was the same as that used in PCRs described in Chapter 4, while in most of RAPD or AP PCR there is a tendency to increase magnesium concentration above 3mM per reaction.

Regarding the specificity of the technique, a number of strains of atypical mycobacteria were included in the analysis of Option 1 of the intersegment PCR. The results (Figure 5.11) indicate the presence of PCR products in all of the MOTTs tested although no similar banding pattern was reproduced in any of the MOTTs (Figure 5.4). In a recently published article, Neimark *et al.* (1996) amplified several DNA fragments from strains of atypical mycobacteria using a similar PCR protocol. Their results concurred with those found in the present study in that the banding pattern was different between strains of the *M. tuberculosis* complex and those of atypical mycobacteria. Concern has been raised recently on the specificity of IS986 for the *M. tuberculosis* complex. Kent *et al.* (1995) detected IS986 related sequences in strains of atypical mycobacteria. It should be noted, however, that these workers did not indicate their criteria for classifying atypical mycobacteria. In the current study, the strains of MOTTs were characterised using protocols described by Watt *et al.* (1993) and their molecular identity confirmed by PCR (Table 4.2), hence the

presence of amplifiable product in these strains confirmed further a suggestion that non-specific priming occurred to sequences outside IS986.

The major application of molecular strain characterisation is the confirmation that related strains are clustered into specific groups by their DNA fingerprints. This is only achievable when DNA elements for typing are polymorphic among unrelated strains. Molecular fingerprinting using PCR has shown great advantages over other technique not only in studies of epidemiology of tuberculosis but in other pathogenic bacteria. For example, Fekete *et al.* (1992) managed to show the heterogeneity among members of the genus *Brucella* by the arbitrarily primed polymerase chain reaction (AP-PCR). They experimented on five different primers, two were 20 mer and the rest 10 mer primers. They were able to show that primers either used individually or in pairs generated banding patterns of epidemiological significance.

A comparison between AP-PCR, ribotyping and monoclonal antibody assay in differentiating strains *Legionella pneumophila* (Gomez-lus *et al.*, 1993) showed that AP-PCR produced better results than those of monoclonal antibody assays. Alos *et al.* (1993) also compared AP-PCR with ribotyping in their study of tracing the nosocomial transmission of *E. coli* K1 in a neonatal unit. Their results showed that AP-PCR produced similar results to ribotyping and led them to demonstrate the nosocomial dissemination.

In view of the above discussion and the fact that the internationally accepted standard DNA typing technique for the *M. tuberculosis* complex (van Embden *et al.*, 1993) was confirmed to be unable to type strains of *M. bovis* (Chapter 4), the intersegment PCR, despite its several shortcomings and testing against only a few laboratory strains, would offer an alternative typing technique for *M. bovis*. This view is supported by a recent report (Gutierrez *et al.*, 1995) where the method as described by Ross and Dwyer (1993) was used to type strains of *M. bovis* from goats and cattle in Spain.

In agreement with other PCR based typing techniques (Table 5.1), and in comparison to other typing techniques i.e. RFLP, PFGE and REA as discussed in Chapter 4, the intersegment PCR fingerprinting offers the advantages of versatility, simplicity and

rapidity, and therefore, has the potential to become a molecular epidemiology tool for typing of *M. bovis*.

5.4.2 DEVELOPMENT OF *M. BOVIS* SPECIFIC PROBE

The quest of many veterinary mycobacteriologists has been to find a *M. bovis* specific antigen and/or DNA marker which could aid studies to better understand the epidemiology of the disease in both man and cattle. So far, a specific probe which could differentiate *M. bovis* from species of the *M. tuberculosis* complex has not been identified. Del Portillo and co-workers (1991) developed a *M. tuberculosis*-specific PCR based on amplification of a single copy gene *mtp40*, which was found only in *M. tuberculosis* and absent in other species belonging to the *M. tuberculosis* complex (Parra *et al.*, 1991). The MPB70 PCR was designed to amplify specifically *M. bovis* (Cousins and Wilton 1992). However, as the results of Chapter 4 have shown, the PCR also amplified the same DNA segment within *M. tuberculosis* strains. In the current study, RAPD PCR was used to generate a number of bands which were found only in *M. bovis* and not in *M. tuberculosis*. RAPD PCR or AP PCR have mostly been used to fingerprint several micro-organisms (see above), but can also be used to generate random DNA fragments which could be used in various purposes as was the case in this experiment. The presence of fragments *f2b* and *f3b* in *M. bovis* only, suggested that they might contain sequences that were unique to the species. Cloning in the pGEM[®]-T vector system and subsequent sequencing revealed that *f2b* comprised 323bp of which 41.5% was GC, whereas *f3b* comprised 184bp and its GC content was 57.6%. This level of GC content was much lower than that estimated for the taxon, the *M. tuberculosis* complex (62.5 to 63.3% - Table 1.4). Putative amino acids sequence found open reading frames in three of six reading frames of *f3b*, suggesting this DNA sequence to be part of a gene sequence. Searches of the EMBL and OWL databases with both DNA and peptide sequences of the two fragments failed to identify meaningful matches. However, searches of the protein database available on the Internet, revealed a 100% homology of the peptide sequence of the third reading frame on the reverse strand of *f3b* to that of a *M.*

tuberculosis cosmid (Database Accession number Z80343). The *M. tuberculosis* showed 75% homology to the *rfbE* gene of *Yersinia enterocolitica* (Database Accession number Z18920). The *rfbE* genes belong to a class of *rfbDEFGH* genes which are essential for O-antigen synthesis (Zhang *et al.*, 1993). O-antigen has been shown to function as a barrier against complement mediated lysis (Joiner *et al.*, and Hackett *et al.*, cited by Zhang *et al.*, 1993) and also plays a role in resistance to killing by microbicidal intracellular granules of polymorphonuclear leukocytes (Stinavage *et al.*, cited by Zhang *et al.*, 1993). Experiments to determine the specificity of the two fragments by screening *PvuII* cleaved DNAs from *M. tuberculosis*, *M. bovis*, *M. terrae* and *M. avium* by the Southern blotting technique, failed to support the putative specificity shown by RAPD PCR. Furthermore, the hybridisation of *f3b* DNA, internal to RAPD primer sequence, to the Southern blots of both *M. bovis* and *M. tuberculosis* resulted in a single band, indicating that these fragments are not representative of repetitive DNA sequences. Searches for the similarity of RAPD primer sequences to the *M. tuberculosis* cosmid failed to demonstrate a perfect match. This observation could explain the reason as to why *f3b* was only amplified in *M. bovis* by RAPD PCR. These findings have underlined the difficulty of differentiating these two species at the genotypic level (Baess 1979; Imaeda 1985), let alone the already known difficulties of phenotypic distinction (Wayne and Kubica 1986).

Several factors were checked on the fragment, including its ability to hybridise with DNA from the pGEM[®]-T vector with and without the insert. The results of this experiment showed that the probe hybridised only with the pGEM[®]-T vector containing the insert and not a pGEM[®]-T vector without the insert (data not shown). Sequence analysis confirmed that the fragment was a RAPD product and not vector sequence. The fragment *f2b* was also confirmed to be a product of RAPD PCR by the sequence as it contained primer sequence.

It can, therefore, be concluded that the techniques developed in the course of this study offered some promise in an attempt to obtain suitable DNA probes for PCR for *M. bovis* and the closely related species *M. tuberculosis*. The suitability of *f3b* sequence for use in identifying strains of *M. bovis* by PCR is yet to be investigated.

However, being a single copy DNA element, makes *f3b* unsuitable for typing strains of *M. bovis*. Further studies need to be performed to determine the phenotypic expression of *f3b* as it has been demonstrated to contain open reading frames in half of its reading frames.

Regarding the intersegment PCR, this technique has the potential to provide alternative means of typing strains of *M. bovis* as well as low IS986 copy number strains of *M. tuberculosis*. But the lack of specificity of the technique necessitates initial identification of the strains by PCR, hence limiting its use on direct typing of the strain from clinical material. Nevertheless, the technique is already in use by other groups who published their work when this work was in progress (Ross and Dwyer 1993; Neimark *et al.*, 1996)

In view of the findings and shortcomings of the current studies, it is not possible to utilise the two developed techniques to type and identify strains of *M. bovis* from Tanzania.

CHAPTER 6

MOLECULAR EPIDEMIOLOGY OF THE *M.*
***TUBERCULOSIS* COMPLEX ORGANISMS IN MAN**
AND CATTLE IN TANZANIA.

CHAPTER 6 MOLECULAR EPIDEMIOLOGY OF THE *M. TUBERCULOSIS* COMPLEX ORGANISMS IN MAN AND CATTLE IN TANZANIA.

6.1. INTRODUCTION

Molecular epidemiology is the integration of molecular techniques to track specific strains of pathogens with conventional epidemiologic approaches to understanding the distribution of diseases in populations (van Embden *et al.*, 1992; Small and Moss 1993).

Molecular techniques described in the preceding chapters are now in place in epidemiological studies of the *M. tuberculosis* complex in man and animals. The justification of utilising molecular techniques in typing *M. tuberculosis* complex organisms arises from several advantages these techniques offer over the conventional phenotyping schemes mentioned in the previous chapters. One molecular technique, widely used in epidemiological studies of the *M. tuberculosis* complex, is the restriction fragment length polymorphism (RFLP), utilising IS986 (IS6110) as a genetic marker (van Embden *et al.*, 1992). IS986, in common with other repetitive elements IS1081, pTBN12, DR, etc., is found in a number of copies within the genome, which provides polymorphisms among unrelated strains. Although these repetitive elements such as IS986 and IS1081, are mobile, they are very highly conserved within the genome, thus offering a stable fingerprint over a period of years, even after several passages (van Embden *et al.*, 1992; Cave *et al.*, 1994). The strongest evidence for the stability of IS986 fingerprint comes from analysis of strains which have been maintained in the laboratory for many decades. *M. tuberculosis* H37R_a was derived from the parental strain H37R_v in 1934 (Skeenken *et al.*, 1934). Despite numerous passages, these strains differ in only one of the nine IS986 copies in both strains (Cave *et al.*, 1991). Thus, only a single transposition occurred over about sixty years of separate propagation. Similar evidence of the stability of IS986 has been shown in *M. bovis* BCG. The work of van Soolingen and co-workers (1991) on strains of *M. bovis* BCG, which originated from a single source and were

distributed to different laboratories world-wide for making BCG vaccine, has shown that all the strains had a single copy of IS986 at the same chromosomal locus, despite the different handling procedures by these laboratories over many years. Furthermore, while antimicrobial resistance of strains of *M. tuberculosis* has been reported to alter with time, the same DNA fingerprint has been maintained by the same strains over a period of time (Rastogi *et al.*, 1992; Thierry *et al.*, 1993; Rigouts and Portaels 1994; Rigouts *et al.*, 1994).

As indicated in the previous chapters, there have been some doubts about the sensitivity of IS986 RFLP typing of strains of *M. bovis* and *M. bovis* BCG, due to the low copy number of the insertion element. Suggestions have been made to overcome this problem by either using a restriction enzyme with a cleavage site within the probe (van Soolingen *et al.*, 1991), thus multiplying by a factor of two the number of bands generated, or use of another repetitive element (Table 1.5a).

The specificity of the fingerprint pattern is dependent upon the copy number of DNA element used, i.e. the number of bands obtained. The higher the copy number, the greater the certainty that two matching patterns represent epidemiologically related strains. In those instances where the patterns contain only a few bands, another probe or method would be of use.

The knowledge acquired from DNA fingerprinting can provide epidemiological data regarding the trends of the disease not only in a single outbreak but on the wider geographical variability of the strains, and on the form of epidemic prevailing. For example, van Soolingen *et al.* (1991) found that the variability of DNA fingerprint patterns is relatively high in countries with low prevalence of the disease. They attributed this to two events; the isolation of strains from people who had been infected and cured at different times in the past, but had experienced a recent re-activation of the latent infection and isolation of strains from new infections acquired from other geographic areas. Low diversity of DNA polymorphisms is found in strains from geographic areas where the majority of tuberculosis cases are caused by the primary infection. In a comparative DNA fingerprinting study carried out by van Soolingen *et al.* (1991) on strains of *M. tuberculosis* from native Dutch people and those from the Central African countries, the investigators found that strains from Dutch cases had a high degree of

polymorphism compared to those from Central African states. Similar observations have been made recently by Hermans *et al.* (1995), who found that there is a small number of predominant families of genetically related strains in Ethiopia and Tunisia despite the endemicity of the disease in those countries, whereas in the Netherlands where tuberculosis is rare many patterns were detected.

Application of molecular techniques for studying of outbreaks of infections caused by the *M. tuberculosis* complex organisms has, nowadays, received wide usage, (Tables 6.1 and 6.2). Although RFLP seems to be the most commonly used technique, other techniques such as REA and PCR typing also have been applied.

A new technique has been introduced recently in the Netherlands by researchers led by Dr. J.D.A. van Embden (Kamerbeek *et al.*, 1993; Schouls *et al.*, 1995). These workers have developed a PCR based typing technique, which takes advantage of the DNA polymorphism at a unique locus in the *M. tuberculosis* chromosome containing multiple 36 base pair direct repeats (DRs). These DRs vary in number and are interspersed by non-repetitive unique spacer sequences, each 35-41 base pairs in length. Because the DR sequence is well conserved among *M. tuberculosis* strains, each DR copy within the locus is a potential target for *in vitro* amplification by PCR and because of the high copy numbers of DRs, such a PCR is more sensitive than using a single copy DNA target. The typing method, designated spoligotyping (spacer oligo typing), relies on the presence or absence of spacer sequences in the amplified DNA which are detected by hybridisation of the labelled amplified DNA to multiple synthetic spacer oligonucleotides which are already bound to a filter. In an initial experiment on a large number of *M. tuberculosis* isolates, these workers found that epidemiologically non-related strains differed in one or more spacers, whereas the isolates from outbreaks were identical (Schouls *et al.*, 1995).

Table 6.1. Selected recent epidemiological studies which have utilised molecular techniques to trace the source or the index case responsible for the outbreak (Studies no. 3, 4, 5, 7, 13 and 14), or establish a clonal relationship of prevailing strains of *M. tuberculosis* (Studies no. 2, 6, 9, 12, 15, 19 and 20).

Reference	Nature of study	Technique(probe) used
1. Rastogi <i>et al.</i> (1992)	Assessment of a single case multiple isolate associated with multiple drug resistance (MDR)	RFLP (IS6110/pTBN12)
2. Godfrey-Faussett <i>et al.</i> (1992)	Genetic fingerprinting of strains from Malawi and Kenya to determine their relatedness	RFLP (IS6110)
3. Genewein <i>et al.</i> (1993)	Molecular approach to identify transmission of tuberculosis in the community	RFLP (IS6110)
4. Coronado <i>et al.</i> (1993)	Transmission of MDR tuberculosis among persons with HIV in an urban hospital	RFLP (pDC73)
5. Jereb <i>et al.</i> (1993)	Nosocomial outbreak of tuberculosis in a renal transplant unit	Mixed-linker PCR
6. Chevrel-Dellagi <i>et al.</i> (1993)	DNA fingerprint of <i>M. tb</i> strains in Tunisia to evaluate the importance of microepidemics	RFLP (IS6110)
7. Takahashi <i>et al.</i> (1993)	A study of an outbreak of <i>M. tuberculosis</i> and <i>M. bovis</i> BCG in Japan	RFLP (IS986)
8. Das <i>et al.</i> (1993)	DNA fingerprinting of sequential isolates of <i>M. tb</i> , before, during and after chemoprophylaxis	RFLP (IS986)
9. Thierry <i>et al.</i> (1993)	Study of unrelated strains from Greece presenting various profiles of drug susceptibility	RFLP (IS6110)
10. Cave <i>et al.</i> (1994)	Assessment of the stability of IS6110 in repeated isolation from cases studied in 4-5 years	RFLP (IS6110)
11. Rigouts <i>et al.</i> (1994)	DNA fingerprinting of strains of drug resistant <i>M. tuberculosis</i> in Czech Republic	RFLP (IS6110)
12. Rigouts and Portaels (1994)	Typing of drug resistant <i>M. tuberculosis</i> strains in Belgium	RFLP (IS6110)
13. Yang <i>et al.</i> (1994)	Evidence of transmission of <i>M. tuberculosis</i> between Greenland and Denmark	RFLP (IS6110)
14. Griffith <i>et al.</i> (1995)	Tuberculosis among health workers in community hospital	PFGE
15. Huh <i>et al.</i> (1995)	Determination of DNA relatedness of strains of <i>M. tb</i> from close contact cases in South Korea	RFLP (IS6110/IS1081)
16. Peerbooms <i>et al.</i> (1995)	Study of Laboratory acquired tuberculosis	RFLP (IS6110)
17. Theisen <i>et al.</i> (1995)	DNA fingerprinting to differentiate mixed strain infection	Mixed-linker PCR
18. van Soolingen <i>et al.</i> (1995)	Typing of <i>M. tuberculosis</i> strains ("the Beijing family") from countries of East Asia	RFLP (IS6110/IS1081), Spoligotyping
19. Yang <i>et al.</i> (1995)	DNA fingerprinting of <i>M. tb</i> from HIV positive and HIV negative patients in Tanzania	RFLP (IS6110)
20. Rigouts <i>et al.</i> (1996)	DNA typing of <i>M. tuberculosis</i> complex strains from Burundi	RFLP (IS6110)

Table 6.2. Application of molecular typing to studies of the epidemiology of *M. bovis* infection in animals and man

Reference	Nature of study	Technique(probe) used
1. Collins <i>et al.</i> (1986)	Typing of <i>M. bovis</i> strains from brush tailed possums in New Zealand	REA
2. Collins <i>et al.</i> (1988)	Investigation of bovine tuberculosis in cattle in New Zealand (possum as a source)	REA
3. de Lisle <i>et al.</i> (1990)	Investigation of <i>M. bovis</i> in cats attending the same veterinary practice in New Zealand	REA
4. Cousins <i>et al.</i> (1993b)	Investigation of an outbreak of <i>M. bovis</i> in captive seals in Western Australia	REA, RFLP (IS6110)
5. Collins <i>et al.</i> (1994a)	Investigation of <i>M. bovis</i> in farmed deer from two outbreaks	REA
6. Collins <i>et al.</i> (1994b)	DNA restriction fragment typing of <i>M. bovis</i> isolates from cattle and badgers in Ireland.	REA
7. Skuce <i>et al.</i> (1994/96)	Genomic fingerprinting of <i>M. bovis</i> from cattle in Northern Ireland	RFLP (IS6110/IS1081/pTBN12)
8. van Soolingen <i>et al.</i> (1994a)	Differentiation of <i>M. bovis</i> strains from animals and man for epidemiology of bovine tuberculosis	RFLP (IS6110/PGRS/DR)
9. Gutierrez <i>et al.</i> (1995)	Differentiation by molecular typing of <i>Mycobacterium bovis</i> strains causing tuberculosis in cattle and goats in Spain.	IS6110 PCR-typing/ RFLP (IS6110/DR/pTBN12)
10. Szwezky <i>et al.</i> (1995)	Molecular epidemiological studies of <i>M. bovis</i> in human and animals in Sweden	RFLP (IS6110)
11. Rigouts <i>et al.</i> (1996)	DNA typing of <i>M. bovis</i> from cattle in Burundi	RFLP (IS6110)
12. Perumaala <i>et al.</i> (1996)	Molecular epidemiology of <i>M. bovis</i> in cattle and deer in U.S.A and Mexico	RFLP (IS6110)

(in Studies 1 and 2 the source was identified, while in the rest, except Study 11, clonal relationship among the strains was established).

A comparison has been made recently between the fingerprints generated by spoligotyping and IS6110 RFLP (van Soolingen *et al.*, 1995) using strains which have been grouped to a single cluster by IS6110 fingerprint pattern, the so called “Beijing family” of *M. tuberculosis* strains. These strains showed a 100% similarity in their spoligotypes, and produced an unusual pattern as they contained only the last 9 of the 43 spacer regions towards the 3’end of the DR cluster.

Limitations to the use of molecular typing in epidemiological studies arise from the following factors: i) type of probe used; for instance, it has been reported that some strains of *M. tuberculosis* from India and East Asia lack IS6110 in their genome despite the fact that those strains appear to be *M. tuberculosis* by biochemical tests (Yuen *et al.*, 1993; van Soolingen *et al.*, 1993; Sahadevan *et al.*, 1995; Thierry *et al.*, 1995), ii) the presence of low copy numbers of IS6110 in the genomes of some of strains of the *M. tuberculosis* complex limit typing of the unrelated strains, especially when the insertion element is integrated at the same chromosomal location (van Soolingen *et al.* 1992) and iii) some repetitive elements, such as IS1081, are found as multiple copies, but offer very little polymorphism in unrelated strains as they are evenly distributed within the genome in all strains of the *M. tuberculosis* complex (Collins *et al.*, 1993).

Molecular strain typing by RFLP analysis also has been found to be useful in identifying laboratory errors, where specimens from suspect patients have been found to be culture positive but the course of the clinical disease disqualified the original diagnosis of tuberculosis. In two separate studies molecular typing techniques were applied and found that laboratory cross contamination had been a source of false positive results (Small *et al.*, 1993; Dunlap *et al.*, 1995). In a study discussed in Chapter 4, a similar approach was able to disprove the isolation of *M. tuberculosis* from cattle tissues.

So far there has been few studies (Table 6.2) where molecular biology techniques have been applied to studies of bovine tuberculosis in cattle and man.

6.1.1. THE AIMS OF THE STUDY

Studies on the epidemiology of bovine tuberculosis in Tanzania, described in Chapters 2 and 3, were intended to establish the zoonotic implication of the disease in human tuberculosis. All the mycobacteria isolates, particularly the *M. tuberculosis* complex, were therefore subjected to molecular analysis. Since the study involved the isolation of strains of the *M. tuberculosis* complex from man, including the isolation of *M. tuberculosis*, the major pathogen of human tuberculosis, the latter organism was bound to cause some difficulties in conventional biochemical strain identification. Therefore, the initial activity of the present study was to confirm the identity of the strains of the *M. tuberculosis* complex using the IS986/*mtp40* multiplex PCR (Sinclair *et al.*, 1995), as well as by a

recently developed *M. bovis* specific PCR (Rodrigues *et al.*, 1995), and, whenever appropriate, other PCRs (Chapter 2) were employed to verify borderline results. Most of the previously reported studies on typing of strains of the *M. tuberculosis* complex (Tables 6.1 and 6.2) have utilised IS6110 RFLP and REA for typing *M. tuberculosis* and *M. bovis* strains, respectively. For the reasons mentioned above, the current study employed IS986 RFLP to type strains of *M. tuberculosis*, whereas pTBN12 RFLP typing was utilised for *M. bovis*. In order to conform with the internationally agreed standard RFLP typing (van Embden *et al.*, 1993) and to determine the diversity of IS986 among the strains of *M. bovis* from Tanzania, some of the blots with DNA from *M. bovis* were stripped of the pTBN12 probe and re-probed with IS986. In order to generate a more diverse fingerprint, and extend the observations in Chapter 3, spoligotyping was applied to all isolates. In spoligotyping, hybridisation of the last 5 of the 43 spacers is found only in *M. tuberculosis* strains and absent in *M. bovis* isolates (van Embden *et al.*, 1995).

6.2. MATERIAL AND METHODS

6.2.1. MYCOBACTERIAL ISOLATES

A total of 102 strains of mycobacteria which were acquired from field investigations in various parts of Tanzania (Chapter 3) were available for this study. This collection included all 44 strains from human cases of tuberculosis and 58 of 103 strains from cattle tissues. It should be noted that 45 strains from tissues of cattle were not analysed as these strains were mainly atypical mycobacteria (n=40) and five *M. bovis* strains that were found be heavily contaminated, precluding a further analysis. The identities of the study strains, as determined at SRML, were as follows: i) human strains; 35 *M. tuberculosis*, 5 *M. bovis*, 2 *M. terrae*, and one each *M. fortuitum* and *M. avium*. ii) cattle strains; 31 *M. bovis*, one *M. tuberculosis*, 10 *M. avium*, 9 *M. terrae*, 3 *M. kansasii*, 2 *M. smegmatis*, one each of *M. fortuitum*, *M. flavescens* and one an unidentified psychrophilic mycobacteria.

The geographical distribution of these strains is presented in Tables 6.3a and b.

6.2.2. GENOMIC DNA EXTRACTION

DNA was extracted from mycobacterial cells grown on either four slopes of IUT and L-J pyruvate or in Middlebrooks 7H9 broths in a similar manner to that described in 2.2.2.2. The latter broth was modified in the later stages of the study as indicated in Chapter 7.

6.2.2. MOLECULAR IDENTIFICATION

The identity of all 102 test strains as, determined by biochemical tests, was confirmed by molecular techniques, specifically IS986/mtp40 multiplex PCR as described in 2.2.2.3.3.5 (Sinclair *et al.*, 1995) and also the recently developed *M. bovis* specific PCR described in 2.2.2.3.3.6 (Rodriguez *et al.*, 1995).

6.2.3. MOLECULAR TYPING

6.2.3.1. Restriction fragment length polymorphism

Southern blotting was carried out on strains of the *M. tuberculosis* complex as described in 2.2.2.4. In order to generate a DNA fingerprint, all *M. tuberculosis* strains were typed using the 245bp fragment of IS986 PCR (2.2.2.3.3.1; Figure 2.2a), while strains of *M. bovis* were typed using pTBN12 (Figure 2.2b). In order to obtain DNA fingerprints (van Embden *et al.*, 1993) some strains of *M. bovis* were probed with the 245bp IS986 PCR product. The probes were labelled as described in 2.2.2.4 for non-radioactive labelling and, whenever appropriate, radioactive labelling was performed as indicated in 2.2.2.4. The latter procedure was performed on blots whenever repeated non-radioactive detection did not provide a good signal on the membranes. Hybridisation of the blots was carried out as described in 2.2.2.4. for non-radioactive and radioactive procedures. Procedure for stripping hybridising signal from the blots was conducted as described in 2.2.2.4.

6.2.3.2. Spoligotyping

All the strains involved in 6.2.3.1 were also typed by this technique as described in 2.2.2.5.

6.2.4. DNA FINGERPRINT ANALYSIS

All RFLP types as well as spoligotype patterns were visually compared and results recorded according to the origin of the strains. Fingerprints were arranged into different types and sub-types on the basis of variation of one or two bands/spacers and whenever the blots had a high level of background bands (as in pTBN12 blots), the major bands were the only ones considered.

The RFLP pattern from IS986 typing was judged on the basis of the number of bands hybridised (van Embden *et al.*, 1993). Due to the presence of numerous bands (>20) generated by pTBN12 RFLP and the lack of a standardised method for interpretation of hybridisation pattern, the RFLP type from pTBN12 RFLP pattern was made on the presence or absence of extra bands in a region between 2.0 to 3.0 Kb and 8.0 to 12.2Kb. Regarding spoligotypes, it was easier to compare the spoligotyping pattern on the basis of recording the spacers without hybridisation starting from spacer number 1 to spacer number 43.

6.2.5. *M. BOVIS* GENETIC RELATEDNESS

As the main thrust of this study was to study the epidemiology of bovine tuberculosis, a need to determine the clonal relationship of the strains of *M. bovis* recovered from animals and man in Tanzania was of paramount importance. The banding patterns deduced from pTBN12 RFLP and spoligotyping were compared and a percentage of genetic relatedness value (GRV) was obtained by using the simplification of the formula described by van Soolingen *et al.* (1991) and Godfrey-Faussett and Stoker (1992).

$$\text{GRV} = \{[(\text{total number of band (spacer) positions shared by patterns} - \text{number of band (spacer) positions different between patterns})] / \text{total number of band (spacer) positions common}\} \times 100.$$

For the pTBN12 RFLP, comparisons were made on bands found on *Pvu*II fragments positioned between 2.0-3.0Kb and 8.0-12.0Kb, while for spoligotypes, the comparison was made on basis of presence or absence of hybridisation signal on all 43 spacers

available on the filters. For simplicity of description, different pTBN12 RFLP type were referred to as clones.

For perfectly matching patterns the GRV=100%, whereas those strains not sharing any common band or spacer GRV=0%.

6.3. RESULTS

6.3.1. MOLECULAR IDENTIFICATION

6.3.1.1. Multiplex IS986/*mtp40* PCR

All 32 cattle strains of the *M. tuberculosis* complex were found to be IS986 positive and *mtp40* negative confirming the identity of the strains as *M. bovis* but not of a single strain of *M. tuberculosis* from cattle (Table 6.3). All 40 human strains of the tuberculosis complex were positive for IS986, while 27 of the 35 strains of *M. tuberculosis* were positive for *mtp40*, the remaining 8 strains of *M. tuberculosis* (all from the Usangu Plains) plus five *M. bovis* strains were negative for *mtp40* (Table 6.3). All 30 strains of atypical mycobacteria from cattle and man were found to be negative by all PCRs, except two of the 11 strains of *M. terrae* which produced a band at approximately 245bp visible on ethidium bromide stained agarose gels (data not shown). This surprising result was cross-checked using IS1081 and MPB70 PCRs and found to be negative. In order to determine the size of the amplified product more accurately, the *M. terrae* multiplex PCR product was analysed using a 8% polyacrylamide gel. In this experiment, the bands produced by the two *M. terrae* strains were found to be below but very close to the 245bp produced by the *M. tuberculosis* complex strains, from which it was concluded that IS986 was specific for the latter group.

6.3.1.2. *M. bovis* specific PCR

The *M. bovis* specific PCR was able to amplify the 495bp product in all 35 strains of *M. bovis* (cattle n=30 and human n=5), and in 31 of the 35 strains of *M. tuberculosis* from man and the single strain from cattle (Tables 6.3).

Table 6.3. Summary of results comparing identification and typing of the *M. tuberculosis* complex strains by standard biochemical tests and molecular methods.

a) Cattle isolates

No.	Strain Ref. No.	Origin	Identity (biochem. tests)	Multiplex PCR		<i>M. bovis</i> PCR	RFLP		Spoligo type	Overall type ^a
				IS986	mtp40		pTBN12	IS986		
C1	M6166	Arusha	<i>M. bovis</i>	√	X	√	A	5A	-	
C2	M6782	Arusha	<i>M. bovis</i>	√	X	√	A	1A	D	1(23) ^b
C3	M6175	Arusha	<i>M. bovis</i>	√	X	√	A	5A	L	2(28)
C4	M6758	Arusha	<i>M. bovis</i>	√	X	√	B	7A	K	3(31)
C5	M6136	Arusha	<i>M. bovis</i>	√	X	√	C		K	4
C6	M6086	Arusha	<i>M. bovis</i>	√	X	√	C		L	5
C7	M6725	Arusha	<i>M. bovis</i>	√	X	√	C	6A	L	5(30)
C8	M6734	Arusha	<i>M. bovis</i>	√	X	√	C	6A	L	5(30)
C9	M6768	Arusha	<i>M. bovis</i>	√	X	√	C		L	5
C10	M6773	Arusha	<i>M. bovis</i>	√	X	√	C	5A	L	5(28)
C11	M6842	Arusha	<i>M. bovis</i>	√	X	√	C		L	5
C12	M6854	Arusha	<i>M. bovis</i>	√	X	√	C		L	5
C13	M6899	Arusha	<i>M. bovis</i>	√	X	√	C		L	5
C14	M6036	Arusha	<i>M. bovis</i>	√	X	√	C	5A	N	6(29)
C15	M6177	Arusha	<i>M. bovis</i>	√	X	√	E		L	7
C16	M6046	Arusha	<i>M. bovis</i>	√	X	√	E	3B	W	8(33)
C17	M6722	Arusha	<i>M. bovis</i>	√	X	√	G	6A	L	9(30)
C18	M6025	Arusha	<i>M. bovis</i>	√	X	√	G	3A	P	10(32)
C19	M7054	Arusha	<i>M. bovis</i>	√	X	√	I	-	L	11
C20	M7128	Arusha	<i>M. bovis</i>	√	X	√	I	-	L	11
C21	M6048	Arusha	<i>M. bovis</i>	√	X	√	L	-	L	12
C22	M9091	Usangu	<i>M. tb</i>	√	X	√	-	-	M	
C23	6252G	Usangu	<i>M. bovis</i>	√	X	√	A	-	-	
C24	M9257	Usangu	<i>M. bovis</i>	√	X	√	A	-	E	13
C25	M9575	Usangu	<i>M. bovis</i>	√	X	√	A	-	E	13
C26	6267B	Usangu	<i>M. bovis</i>	√	X	√	A	-	E	13
C27	M9551	Usangu	<i>M. bovis</i>	√	X	√	A	-	X	14
C28	5861L	Usangu	<i>M. bovis</i>	√	X	√	D	-	E	15
C29	M9070	Usangu	<i>M. bovis</i>	√	X	√	F	6A	L	16(30)
C30	M8837	Usangu	<i>M. bovis</i>	√	X	-	G	-	-	
C31	M9078	Usangu	<i>M. bovis</i>	√	X	√	K	6A	L	17(30)
C32	M8484	Usangu	<i>M. bovis</i>	√	X	√	M	1A	C	18(34)

√ positive X negative - test not done

^acombined pTBN12 & spoligotyping ^bcombined IS986 & spoligotyping (see Table 6.3b).

Table 6.3 continued

b) Human isolates

No.	Strain Ref. No.	Source	Identity	Multiplex PCR		<i>M. bovis</i> PCR	RFLP		Spoligo type	Overall type ^b
				IS986	<i>mtp40</i>		IS986	pTBN12		
M1	TB6/1/94	Arusha	<i>M. tb</i>	√	√	-	13A	-	-	
M2	TB4/3/93	Arusha	<i>M. tb</i>	√	√	√	13A	-	T	1
M3	TB5/3/93	Arusha	<i>M. tb</i>	√	√	√	13A	-	T	1
M4	TB1/7/93	Arusha	<i>M. tb</i>	√	√	X	13A	-	T	1
M5	TB2/7/93	Arusha	<i>M. tb</i>	√	√	X	13A	-	T	1
M6	TB4/1/94	Arusha	<i>M. tb</i>	√	√	√	13D	-	H	2
M7	TB5/1/94	Arusha	<i>M. tb</i>	√	√	√	13F	-	Z	3
M8	TB8/1/94	Arusha	<i>M. tb</i>	√	√	√	14A	-	T	4
M9	TB3/1/94	Arusha	<i>M. bovis</i>	√	X	√	5A	G	K	5(19) ^c
M10	TB7/1/94	Arusha	<i>M. bovis</i>	√	X	√	9B	J	Y	6(20)
M11	TB5/1/95	MMC ^a	<i>M. bovis</i>	√	X	√	-	C	U	(21)
M12	TB9/1/95	MMC	<i>M. tb</i>	√	√	√	10B	-	S	7
M13	TB6/1/95	MMC	<i>M. tb</i>	√	√	√	14A	-	T	4
M14	TB11/11/95	MMC	<i>M. tb</i>	√	√	√	1B	-	AC	8
M15	TB12/2/95	MMC	<i>M. tb</i>	√	√	√	4B	-	F	9
M16	TB17/2/95	Usangu	<i>M. tb</i>	√	X	√	-	-	O	
M17	TB19/2/95	Usangu	<i>M. tb</i>	√	√	√	-	-	Q	
M18	TB22/9/94	Usangu	<i>M. tb</i>	√	√	√	10A	-	I	10
M19	TB5/11/95	Usangu	<i>M. tb</i>	√	√	√	10C	-	AA	11
M20	TB21/1/95	Usangu	<i>M. tb</i>	√	√	√	10D	-	I	12
M21	TB17/5/95	Usangu	<i>M. tb</i>	√	√	√	12A	-	B	13
M23	TB18/2/95	Usangu	<i>M. tb</i>	√	X	√	12B	-	R	14
M24	TB15/2/95	Usangu	<i>M. tb</i>	√	X	√	12B	-	S	15
M25	TB13/1/95	Usangu	<i>M. tb</i>	√	X	√	12B	-	V	16
M26	TB23/1/95	Usangu	<i>M. tb</i>	√	√	√	12B	-	Z	17
M27	TB2/7/94	Usangu	<i>M. bovis</i>	√	X	√	13B	H	AA	18(22)
M28	TB1/7/94	Usangu	<i>M. tb</i>	√	√	√	13C	-	T	19
M29	TB1/2/95	Usangu	<i>M. tb</i>	√	X	√	13E	-	T	20
M30	TB18/1/95	Usangu	<i>M. tb</i>	√	X	√	13E	-	T	20
M31	TB1/5/94	Usangu	<i>M. tb</i>	√	√	√	15A	-	T	21
M32	TB3/10/94	Usangu	<i>M. tb</i>	√	X	√	15A	-	T	21
M33	TB16/9/94	Usangu	<i>M. bovis</i>	√	X	√	15A	H	T	21(23)
M34	TB3/5/94	Usangu	<i>M. tb</i>	√	√	√	15B	-	T	22
M35	TB20/2/95	Usangu	<i>M. tb</i>	√	X	√	1A	-	D	23
M36	TB20/1/95	Usangu	<i>M. tb</i>	√	√	√	1B	-	AB	24
M37	TB21/9/94	Usangu	<i>M. tb</i>	√	√	√	4A	-	A	25
M39	TB24/5/95	Usangu	<i>M. tb</i>	√	√	√	7A	-	G	26
M40	TB0/9/94	Usangu	<i>M. tb</i>	√	√	√	9A	-	J	27

^a MMC - Muhimbili Medical Centre, Dar-es-Salaam. √ positive X negative

- test not done ^bcombined IS986 & spoligotyping

^ccombined pTBN12 & spoligotyping (see Table 6.3a).

The validity of the results on *M. bovis* specific PCR were checked by using the template DNA which was used earlier for the spoligotyping and results were reproducible in all doubtful strains. It should be noted that this technique was not applied to any of the atypical mycobacteria due to limitation of time.

6.3.2. MOLECULAR TYPING

6.3.2.1. RFLP analysis of *M. bovis* strains using pTBN12 as a genetic marker

Regarding pTBN12 typing, all 35 strains of *M. bovis* from cattle (30) and man (5) were found to belong to 13 different pTBN12 RFLP patterns (Table 6.3). On the basis of the similarity of the banding patterns, more than half of the strains fell into two major groups i.e. type A and C, with eight and eleven strains respectively (Table 6.3). The typical patterns are shown in Figure 6.1. pTBN12 RFLP types G and C were common in cattle and human isolates of *M. bovis*, while RFLP types H and J were found exclusively in human strains, and the rest of the RFLP types (A,B,D,E,F,I,K,L &M) were only from cattle isolates (Figure 6.2). Geographical distribution of pTBN12 RFLP types (Figure 6.3) shows that type A was in 50% and 14% of strains of *M. bovis* recovered from the Usangu Plains and Arusha respectively, while pTBN12 RFLP type C was mainly confined to strains of *M. bovis* from Arusha (47.6%).

6.3.2.2. RFLP analysis of the *M. tuberculosis* complex strains using IS986 as a genetic marker

IS986 RFLP patterns (Figure 6.4) revealed that strains of the *M. tuberculosis* complex had IS986 copy number ranging from 1 to 15. The RFLP types 1A, 5A and 7A were shared by strains of *M. bovis* recovered from cattle and man, while RFLP types 3A, 3B and 6A were exclusive to *M. bovis* from cattle and the remaining RFLP types (1B, 4A, 4B, 9A-B, 10A-D, 13A-F, 14A, and 15A-B) were found only amongst *M. tuberculosis* and *M. bovis* from man (Table 6.3; Figure 6.5).

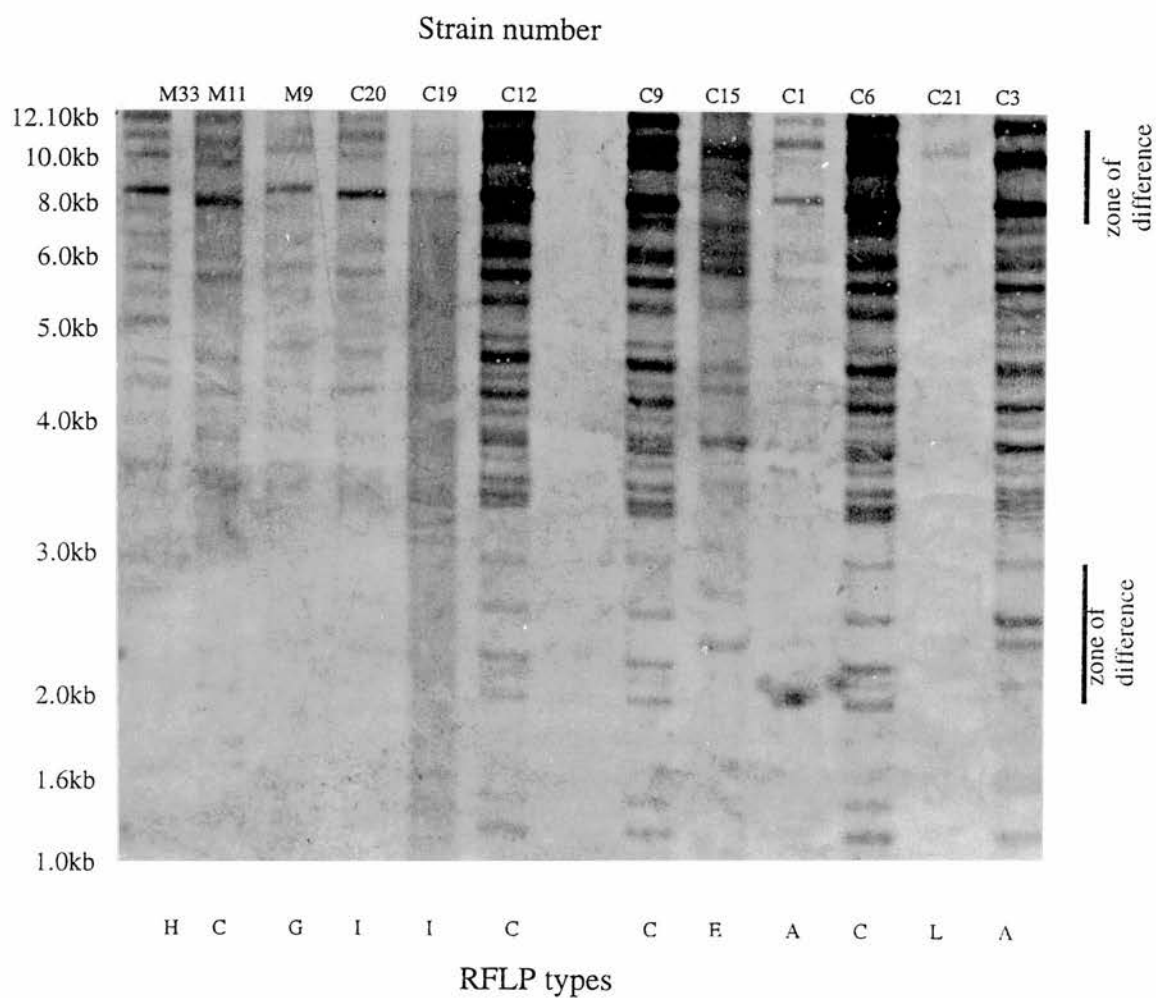


Figure 6.1. The pTBN12 RFLP fingerprint patterns for strains of *M. bovis* from cattle and man. Numbers on the left indicate molecular weights in kilobase pairs.

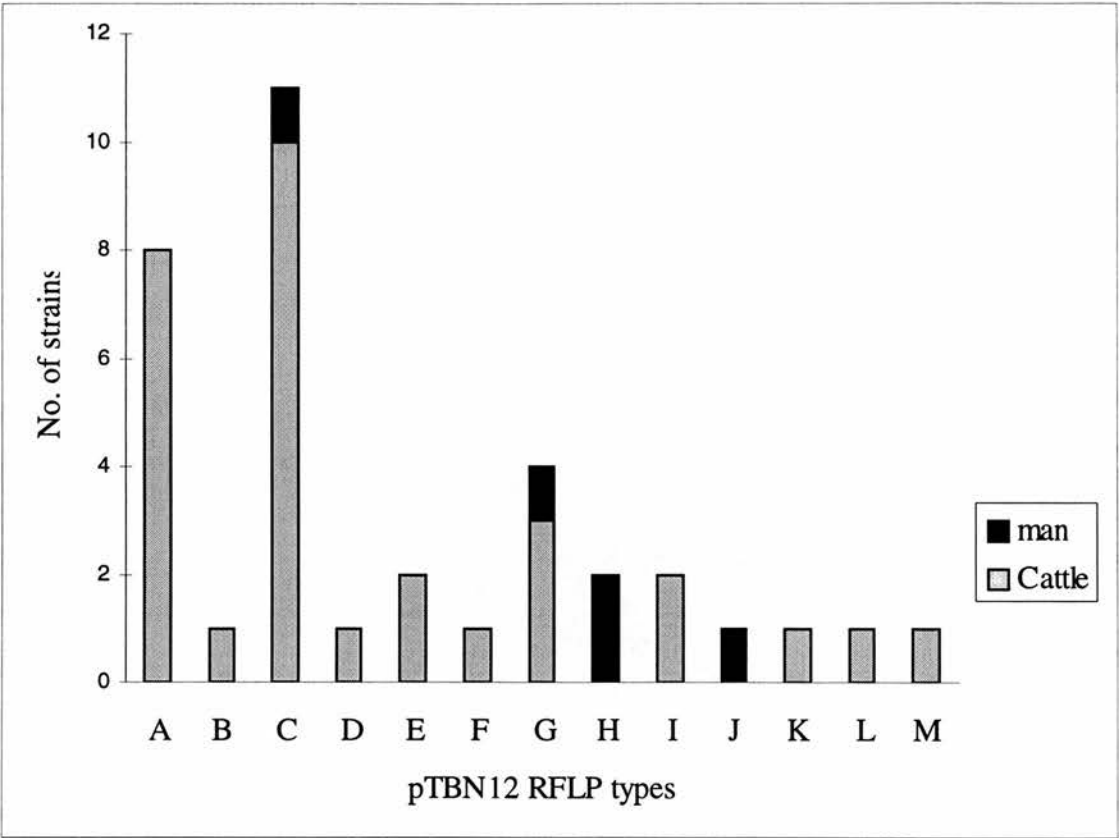


Figure 6.2. The pTBN12 RFLP types for strains of *M. bovis* from cattle and man

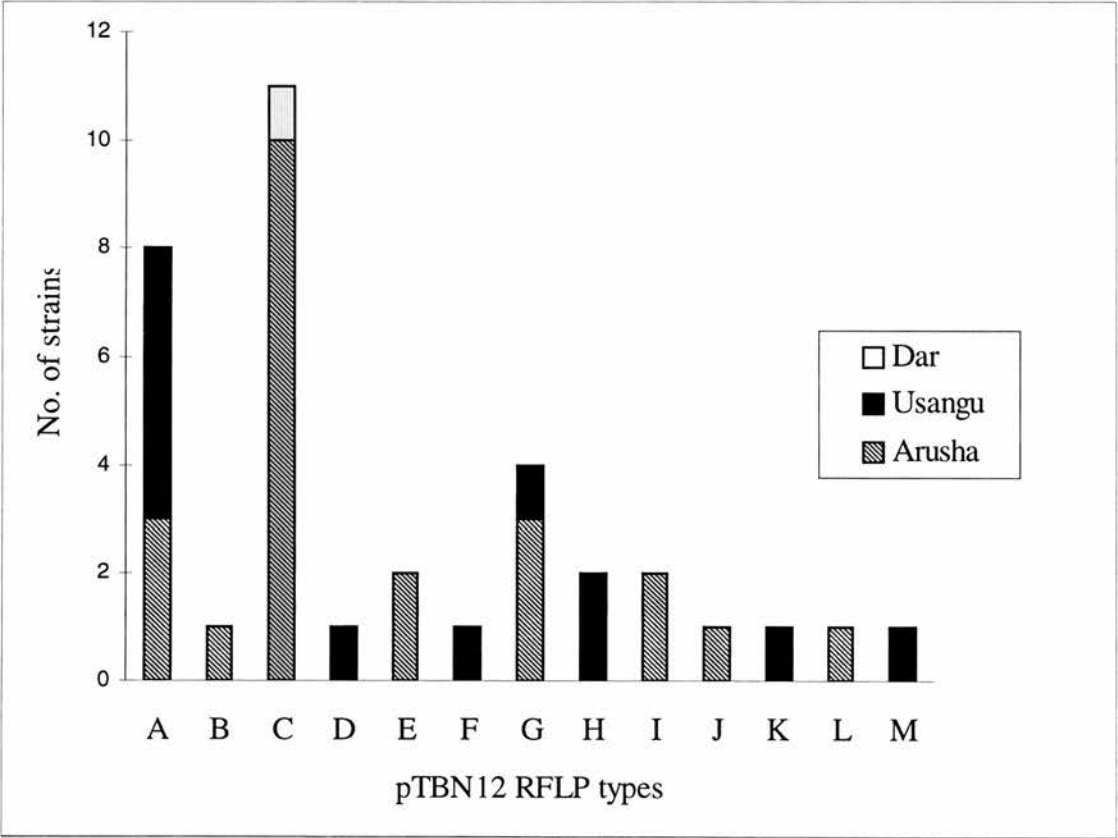


Figure 6.3. The geographical distribution of the pTBN12 RFLP types for strains of *M. bovis*.

The distribution of the RFLP types amongst strains from different areas of the study (Figure 6.6) shows some RFLPs were found in more than one location. For example, RFLP type 1B was found amongst strains from Usangu area and Dar-es-Salaam, type 14A found in isolates from Dar-es-Salaam and Arusha and RFLP types 1A, 6A and 7A was common to isolates from the Usangu and Arusha areas. All four *M. bovis* from man gave different IS986 RFLP types. Two strains of *M. bovis* from the Usangu Plains were found to have a high copy number of IS986 (i.e. 13 and 15) and a similar banding pattern (15A) was also found in *M. tuberculosis* from the same geographical area.

The remaining RFLP types were confined to particular geographical areas (Figure 6.6). The eight strains of *M. tuberculosis* from Arusha (Table 6.3) were found to contain a genetically less heterogeneous population which contained between 13 and 14 IS986 copies with most of these (62.5%) strains being IS986 RFLP type 13A. A total of 11 RFLP types were encountered in Arusha strains of the *M. tuberculosis* complex. RFLP types 5A, 6A and 13A were found in more than a single strain. Strains from the Usangu Plains showed a more polymorphic DNA fingerprint profile. There were 17 different RFLP types, with types 12B, 13E and 15A being found in more than one case. The copy number of IS986 varied from as low as 1 to as high as 15. Strains from Dar-es-Salaam, though few, had a very different DNA profile amongst them, with the copy number of IS986 varying from 1-14.

In order to determine the suitability of IS986 as a probe for typing of *M. bovis* from Tanzania. The RFLP of these 18 *M. bovis* were compared with those of 31 strains of *M. tuberculosis* (Figure 6.7). It was revealed that these isolates of *M. tuberculosis* fell into 19 while those of *M. bovis* had 10 different IS986 RFLP patterns. RFLP types 1A, 7A and 15A were common to both species, while the remaining types were found in either *M. tuberculosis* or *M. bovis* (Figure 6.7). Table 6.4. summarizes the distribution of IS986 copy number of strains of *M. tuberculosis* and *M. bovis* from man and cattle.

One strain of *M. bovis* from a human patient in Arusha gave a similar IS986 RFLP type (5A) to that found in *M. bovis* from cattle within the same geographical area (Table 6.3). Of the 14 *M. bovis* from cattle, two major clones were identified, IS986 RFLP types 5A

and 6A comprising of 28.6% and 35.7% of the 14 *M. bovis* isolates analysed (Figure 6.5).

Although only a few strains from the Usangu Plains were typed using this probe, IS986 RFLP types found among those strains were similar to those found in *M. bovis* strains from Arusha (Table 6.3).

There were 5 strains (3 *M. tuberculosis* and 2 *M. bovis*) with single copy of IS986 which was integrated on either a 1.9Kb, 4.5Kb or 5.0Kb *Pvu*II restriction fragment from genomic DNA.

Strains of *M. tuberculosis* and *M. bovis* were clustered into three groups according to the number of copies of IS986 they contained as follows: 1-5 copies, Cluster I; 6-10 copies, Cluster II; and 11-15 copies, Cluster III. The categorisation of strains of *M. tuberculosis* and *M. bovis* into the three main clusters shows that Cluster III was the dominant cluster with 64.5% of the total *M. tuberculosis* isolates classified, while in *M. bovis* the majority of the strains were in Cluster I (50%), followed by Cluster II (38.9%) (Table 6.4).

Table 6.4. The categorisation of strains of the *M. tuberculosis* complex according to different IS986 RFLP clusters

	IS986 RFLP Cluster (copy number)			Total
	I (1-5)	II (6-10)	III (11-15)	
<i>M. tuberculosis</i> (n= 31)				
Number of RFLP types	4	4	10	18
Number of strains	5	6	20	31
<i>M. bovis</i> (n= 18)				
Number of RFLP types	5	3	2	10
Number of strains	9[1]*	7[1]	2[2]	18[4]

* number of human *M. bovis* strains

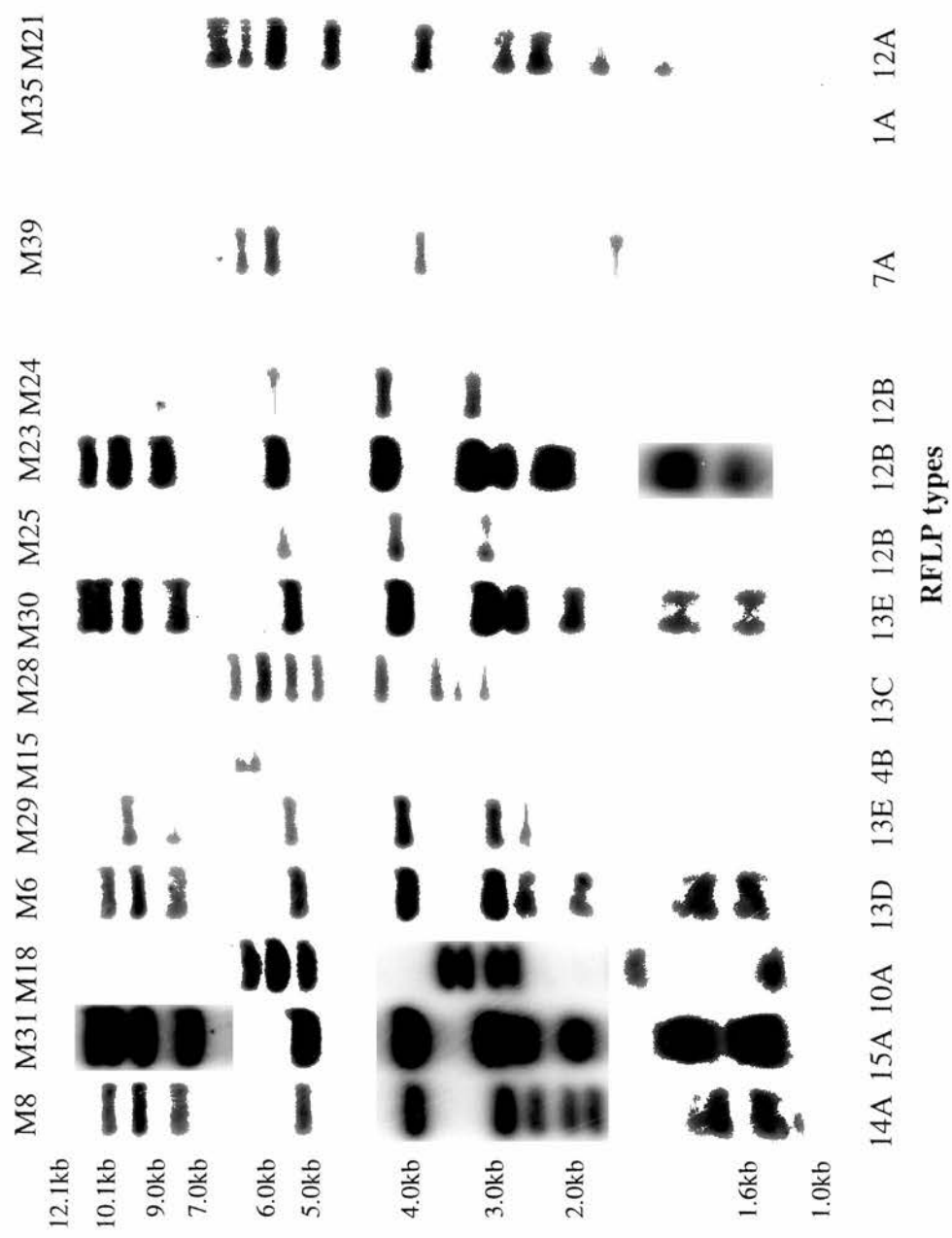


Figure 6.4. The IS986 RFLP fingerprint patterns for the strains of the *M. tuberculosis* complex from man. Numbers on the left indicate the molecular weights in kilobase pairs.

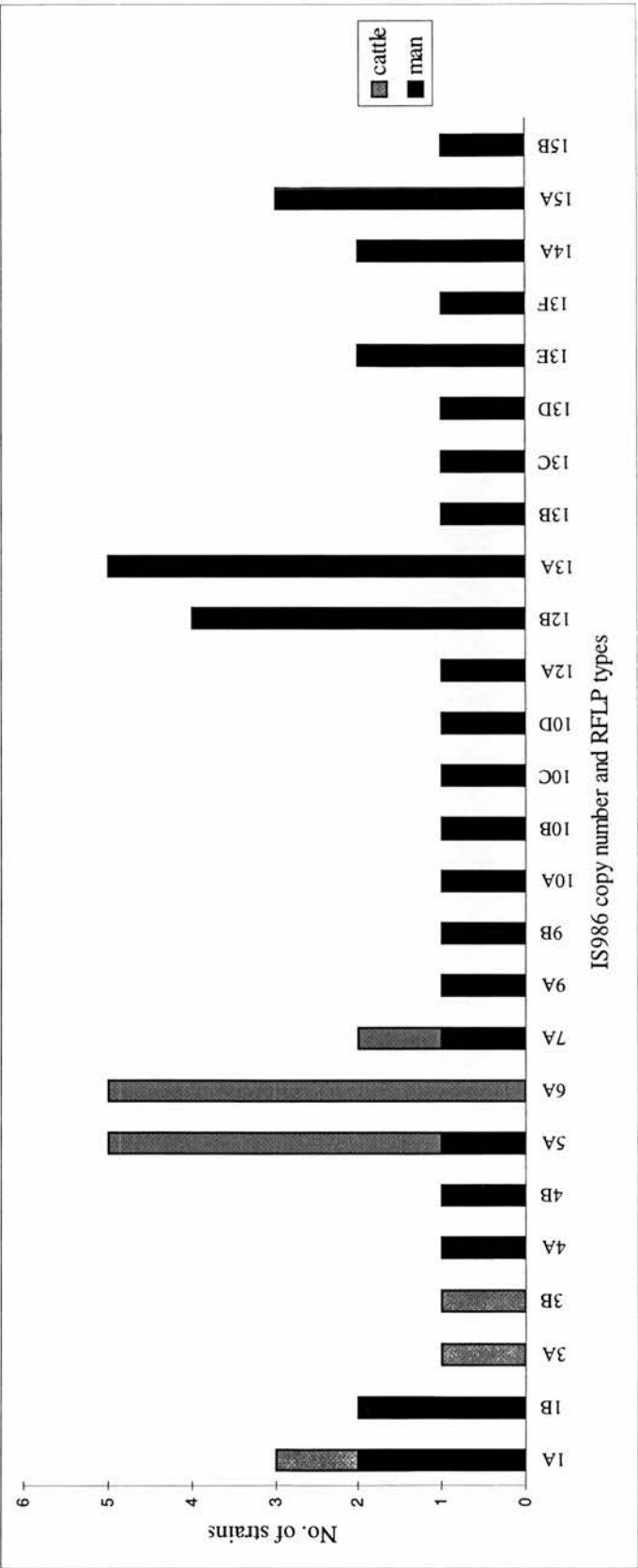


Figure 6.5. The distribution in cattle and man of IS986 RFLP types for isolates of the *M. tuberculosis* complex

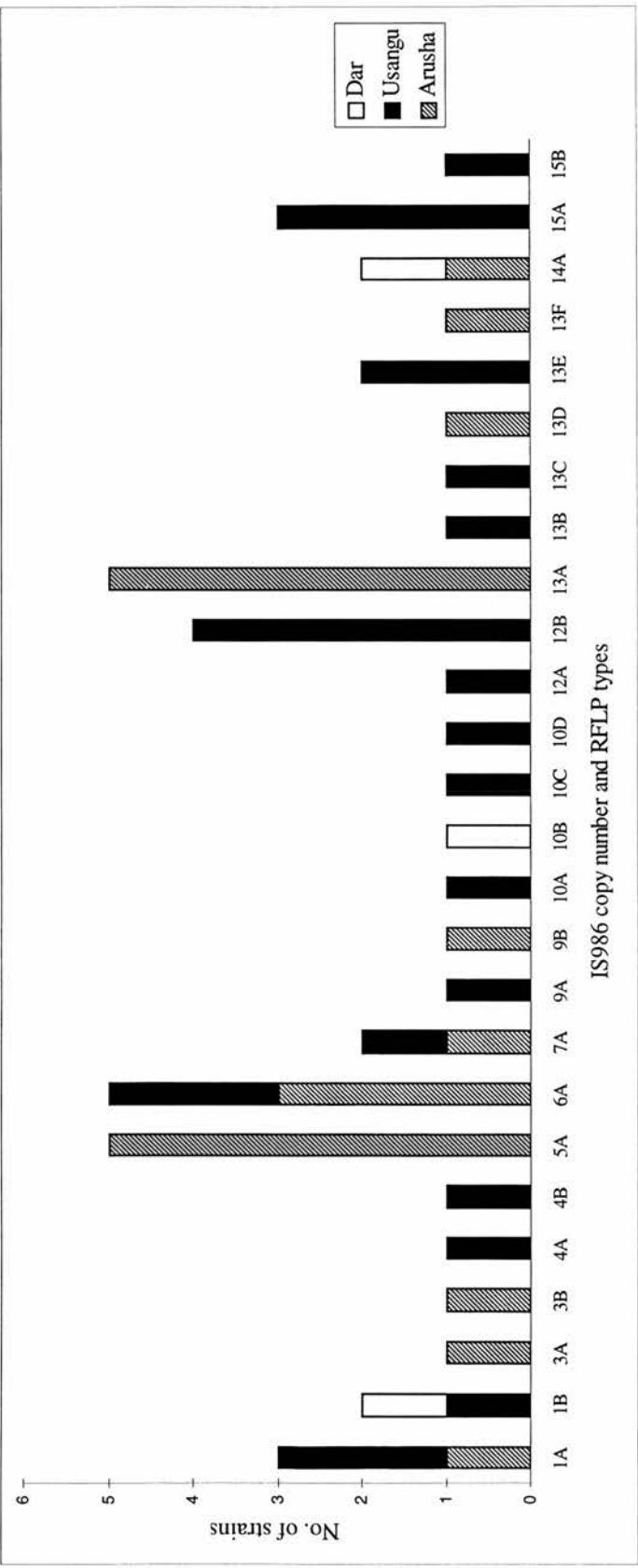


Figure 6.6. The geographical distribution of IS986 RFLP types for strains of *M. tuberculosis* complex.

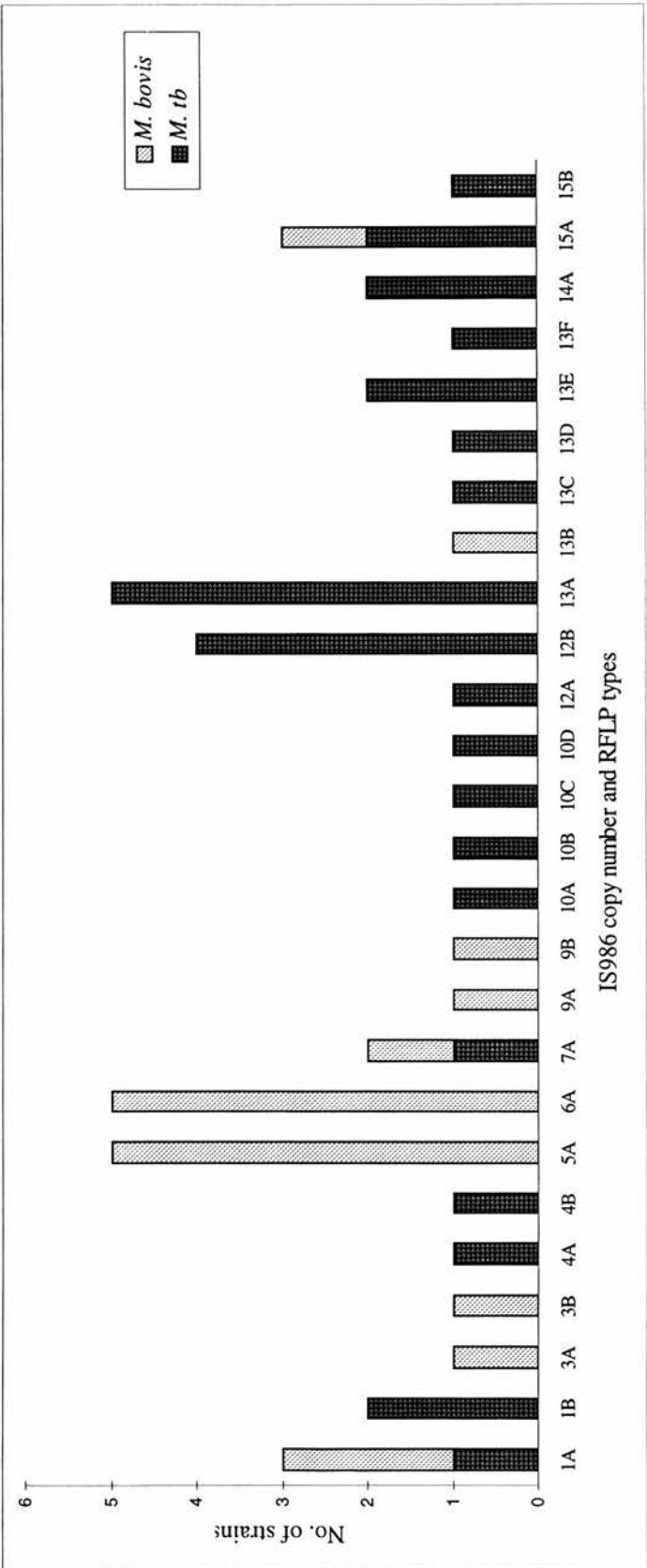


Figure 6.7. The distribution of the IS986 RFLP types among strains of the *M. tuberculosis* and *M. bovis*.

6.3.2.3. DNA polymorphism of strains of *M. tuberculosis* and *M. bovis* as determined by spoligotyping technique.

29 different spoligotypes were identified in the 68 strains of *M. tuberculosis* complex examined (Table 6.3; Figure 6.8). 19 of the 29 Spoligotypes were present only in isolates from man, while only 8 of 29 spoligotypes were confined to strains from cattle (Figure 6.9). Only two of the 29 spoligotypes were shared by strains of *M. tuberculosis* complex found in man and cattle (Figure 6.9). The dominant spoligotype in strains from cattle was L (n=15), mostly *M. bovis*, while the dominant spoligotype amongst strains from man was type T (n=12), which was found in 11 *M. tuberculosis* and one *M. bovis* isolate (Figure 6.10).

Differentiation of strains of the *M. tuberculosis* complex using this technique was able to classify isolates of *M. tuberculosis* and *M. bovis* into 16 and 10 unique spoligotype respectively (Figure 6.10). There were three spoligotypes found in both species of *Mycobacterium* (Spoligotype D, T and AA). Only nine of 29 spoligotypes (D, E, I, K, L, S, T, Z & AA) were found in more than a single isolate, the remaining 20 spoligotypes were present in single isolates (Figure 6.11).

Regarding the geographical distribution of spoligotypes in the study area. The summary (Figure 6.11) shows that Spoligotype T was found in all parts of the country. Two strains each from Dar es Salaam and Usangu shared the same spoligotype patterns S and X, while strains from Usangu and Arusha were similar in Spoligotypes D, L and Z (Figure 6.11). The remaining 23 spoligotypes were unique to specific regions (Usangu 14; Arusha 7; Dar es Salaam 2 - Figure 6.11).

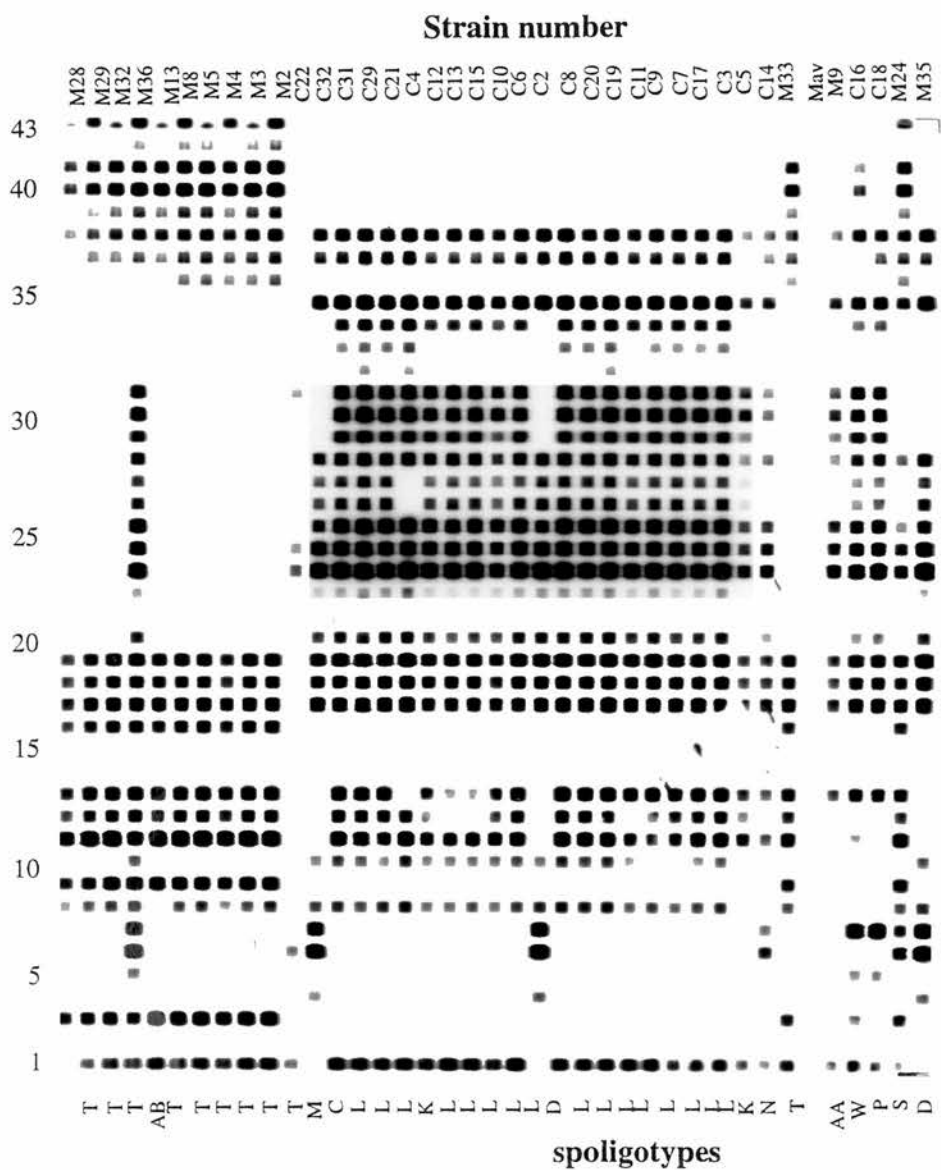


Figure 6.8. The spoligotype patterns for the strains of the *M. tuberculosis* complex from man and cattle, Spacer number are indicated on the left (Mav = *M. avium* - negative control).

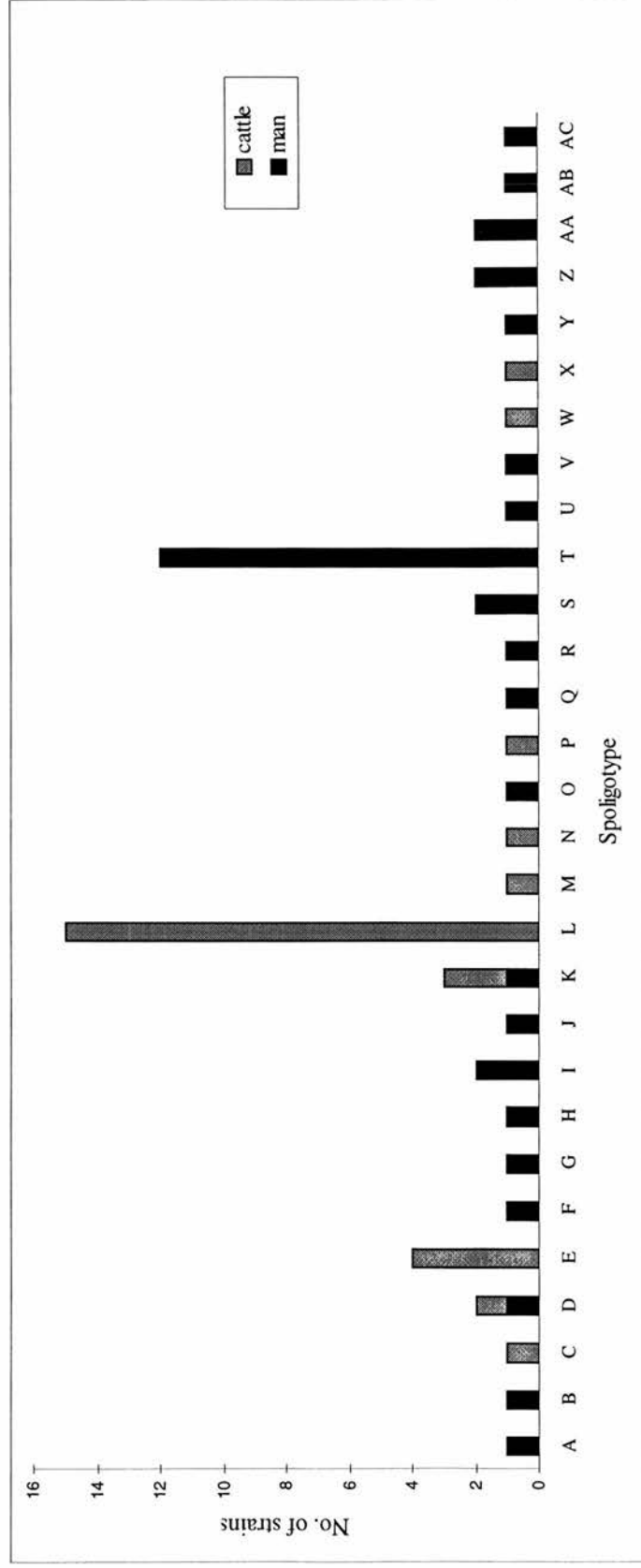


Figure 6.9. The distribution of spoligotypes for isolates of the *M. tuberculosis* complex from cattle and man

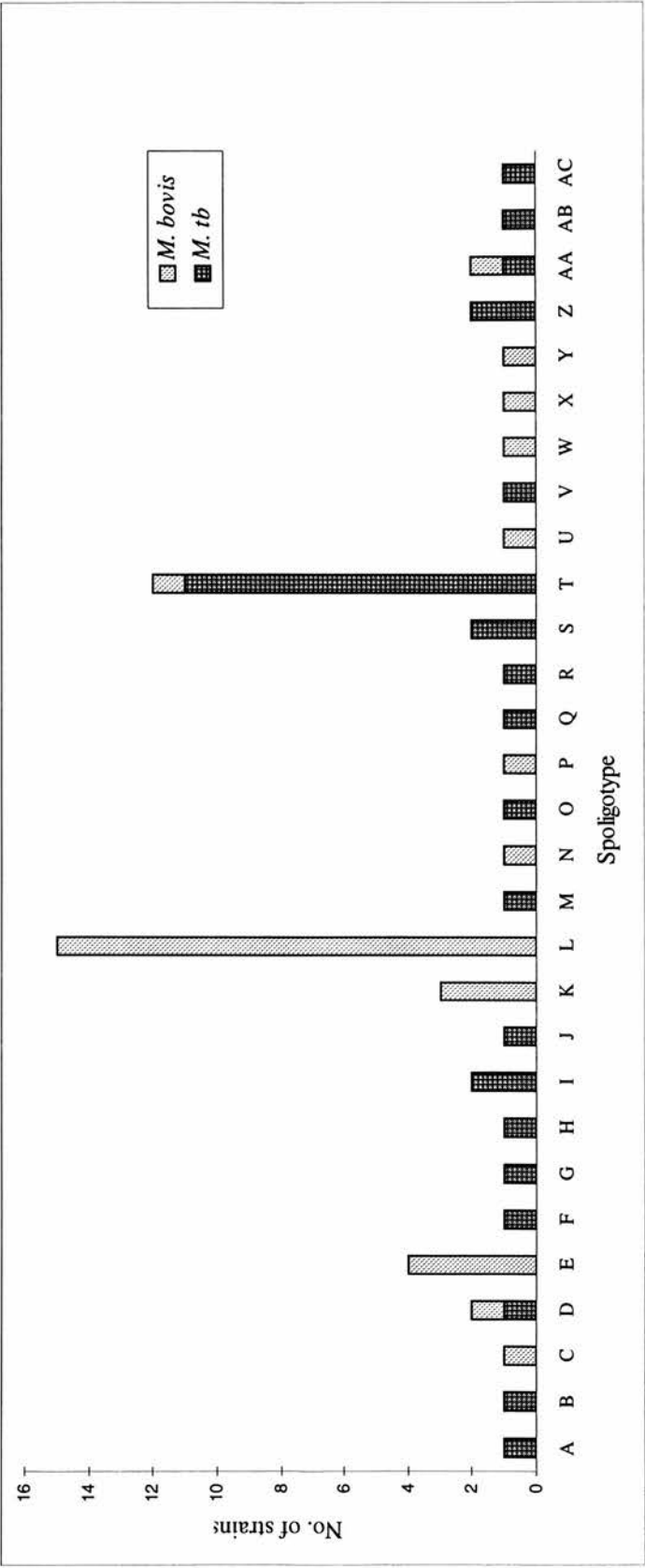


Figure 6.10. The distribution of the spoligotypes among strains of the *M. tuberculosis* complex.

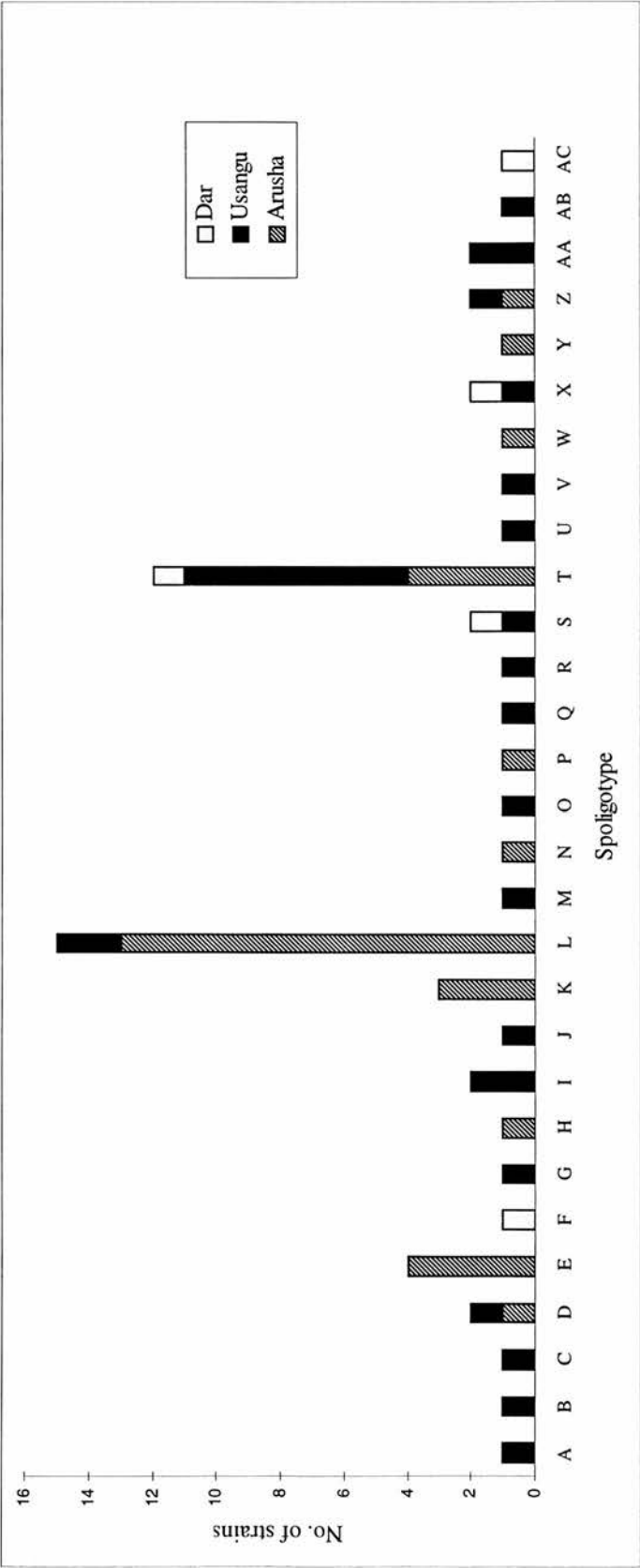


Figure 6.11. The geographical distribution of spoligotypes types for strains of the *M. tuberculosis* complex.

6.3.2.4. Comparison of RFLP and spoligotype

In order to simplify analysis, comparison of fingerprints generated by the two DNA fingerprinting techniques i.e. RFLP and spoligotyping, to characterise strains of the *M. tuberculosis* complex from Tanzania (Figure 6.12) was restricted to RFLP types found in three or more strains. Using this criterion, comparison could be made on strains with pTBN12 RFLP types A, and C (type G had only two of the three strains spoligotyped), and IS986 RFLP types 1A, 5A, 6A, 12B, 3A, 15A (Figure 6.12; Table 6.3). Although the number available for comparison were small, it was apparent that there was some agreement between the typing techniques.

pTBN12 RFLP types found in strains of *M. bovis* revealed a similar spoligotype in 50 to 75% of the strains analysed (Figures 6. 12 a & b). It should be noted that 50% of the strains of *M. bovis* which had the major spoligotype (type L) were pTBN12 RFLP type C and all strains of *M. bovis* with spoligotype E were pTBN12 RFLP type A (Table 6.3).

The relationship between IS986 and spoligotyping was less clear, with the exception of RFLP types 6A, 13A and 15A, where there was total agreement with spoligotypes L, T and T respectively (Figure 6.12). All the strains of the major spoligotype (T) for *M. tuberculosis* were those belonging to dominant cluster III (IS986 RFLP types 13A, 13C, 13E, 14A, 15A & 15B) (Table 6.3b).

The result of combining RFLP and spoligotype patterns categorised strains of the *M. tuberculosis* complex into more numerous DNA fingerprints than by an individual technique (Table 6.3). For instance, 28 strains from cattle and 5 from man typed by pTBN12 RFLP and spoligotyping, produced 13 pTBN12 RFLP types and 12 spoligotypes respectively, but by combining the two they were classified into 23 distinct DNA fingerprint patterns (Table 6.3). 36 strains from man and 13 from cattle typed by IS986 RFLP and spoligotyping were categorised into 26 IS986 RFLP types and 23 spoligotype patterns, whereas the combination of the two resulted to 34 distinct DNA fingerprint patterns (Table 6.3).

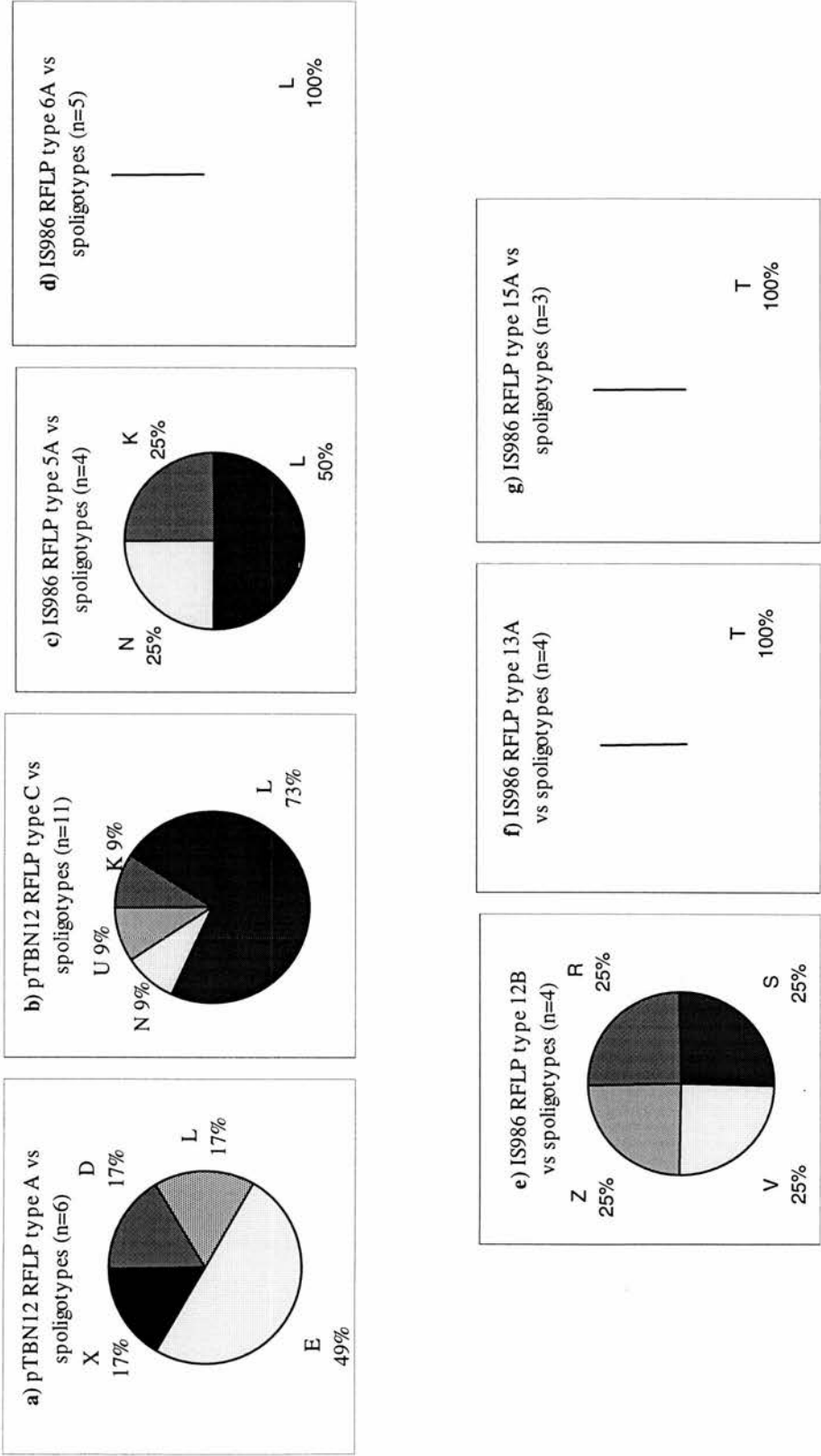


Figure 6.12. The comparison of RFLP types (a & b pTBN12; c-g IS986) to corresponding spoligotypes in strains of the *M. tuberculosis* complex.

6.3.3. THE GENETIC RELATEDNESS OF STRAINS OF *M. BOVIS* ISOLATED IN TANZANIA

The genetic relationship of *M. bovis* strains recovered from the study areas in Tanzania varied from as low as 29% between strains of Clone F and M to as high as 86% in between strains of Clone A and Clones B, C and D; Clone C and Clones G and I; Clone D and Clones G and H; Clone E and Clone J; Clone H and Clone J, as identified by pTBN12 RFLP (Table 6.5a). The dominant patterns (Types A and C) found in the two geographical areas under study were 86% related. The pTBN12 RFLP types found in human strains of *M. bovis* (Types C, G, H & J) were on average related by 71% (range 57 to 86%) to Type A, whilst their relatedness to Type C was on average 82% (range 71 to 100%) (Table 6.5a). The pTBN12 RFLP types found in strains recovered from the Usangu Plains (A,D,F,G,K,M&H) were related by between 29 to 86%, while the genetic similarity those from Arusha (A,B,C,E,G,I,J&L) ranged from 43% to 86% (Table 6.5a).

As regards the genetic relatedness determined by spoligotypes the similarity ranged from 51% (Spoligotype M vs W) to 98% (Spoligotypes X, Y and L) (Table 6.5b). The spoligotypes encountered in *M. bovis* from human cases were K, T, U, Y and AA, these strains were genetically similar to the main spoligotype of *M. bovis* from cattle (type L) as follows; 93%, 60%, 65%, 98% and 70% respectively. Spoligotype E was the dominant type amongst the strains from Usangu Plains. The isolates with this pattern were genetically related to other strains from the same geographical area by an average of 79% (range 74 and 95%). 14 of 20 isolates of *M. bovis* from Arusha were spoligotype pattern L. The average relatedness of this group to the rest of strains was found to be 85% (range 74 to 93%).

Table 6.5a. The genetic relatedness value (GRV) amongst pTBN12 RFLP types of *M. bovis* isolates from Tanzania.

	pTBN12 RFLP types (Clones)											
	A	B	C	D	E	F	G	H	I	J	K	L
B	86											
C	86	71										
D	86	71	71									
E	71	57	71	71								
F	71	57	57	57	57							
G	71	57	86	86	71	57						
H	71	57	71	86	57	71	71					
I	57	57	86	71	57	43	71	71				
J	57	43	71	43	86	71	57	86	43			
K	57	57	71	57	57	71	57	43	43	71		
L	57	71	71	43	57	43	57	43	57	43	57	
M	43	43	57	57	57	29	71	57	71	43	57	43





 86% Highest level of genetic relatedness
 29% Lowest level of genetic relatedness

Table 6.5b. The genetic relatedness values (GRV) amongst spoligotypes of *M. bovis* isolates from Tanzania.

	Spoligotypes												
	L	C	D	E	K	M	N	P	T	U	W	X	Y
C	79												
D	81	95											
E	73	95	93										
K	93	79	81	77									
M	65	67	67	74	70								
N	93	79	95	79	77	70							
P	84	77	79	77	86	67	84						
T	60	67	67	65	67	72	65	58					
U	65	74	74	72	70	67	67	58	91				
W	74	56	60	53	70	51	70	74	60	58			
X	98	74	79	74	91	63	91	86	58	63	74		
Y	98	74	79	74	91	63	91	84	58	63	74	98	
AA	70	65	70	65	65	53	70	63	74	74	77	70	72

 98% Highest level of genetic relatedness
 51% Lowest level of genetic relatedness

6.4. DISCUSSION

The present study has for the first time demonstrated the zoonotic importance of *M. bovis* infection in man and cattle in Tanzania by the use of molecular techniques. Although there were few strains of *M. bovis* from man the DNA fingerprints of some of the strains were found to be similar to those found in cattle strains by individual typing techniques. There was a lack of agreement in between individual typing techniques which underline the need to standardize the molecular typing techniques for *M. bovis*.

6.4.1. MOLECULAR IDENTIFICATION

In the course of the current study, strains of the *M. tuberculosis* complex were initially identified by the use of biochemical tests (Watt *et al.*, 1993). These tests are cumbersome to perform and rely on lengthy cultural procedures, therefore, all the strains recovered from cattle and man in Tanzania were subjected to a PCR-based identification.

The nucleic acid amplification based methods for the identification of mycobacteria fall into one of the three options: i) genus specific PCR, ii) taxon specific PCR and iii) species specific PCR. A wide range of PCR protocols are now available for the first two options. The third option has been successful to a limited extent in studies involving members of the *M. tuberculosis* complex. The IS986/mtp40 multiplex PCR developed in the course of these studies (Sinclair *et al.*, 1995) was used to first of all differentiate members of the tuberculosis complex from other mycobacteria and secondly to discriminate *M. tuberculosis* from other members of the group, particularly *M. bovis*. During the execution of these studies, an article describing a *M. bovis* specific PCR was published (Rodriguez *et al.*, 1995) hence this protocol was also used to differentiate *M. bovis* from *M. tuberculosis*. It was essential to clearly identify the two strains as the subsequent molecular typing technique utilised different probes. Strains of *M. bovis* were typed by pTBN12 while those of *M. tuberculosis* were analysed by IS986.

The results of the present study (Table 6.3) have shown the ability of the IS986 component within the multiplex PCR to identify strains of the *M. tuberculosis* complex. Although two *M. terrae* strains produced a band of approximately 245bp, this product was shown not to be IS986, confirming that the environmental mycobacteria tested here were negative to this PCR and indicating a high degree of specificity of the protocol. Contradictory findings have reported indicating that *M. terrae* can give apparently positive reactions with the *M. tuberculosis* complex DNA probes. Ford *et al.* (1993) and Emler *et al.* (1995) found the positive result to be due to the use of a probe derived from 16SrRNA gene sequences which are common to both *M. terrae* and the *M. tuberculosis* complex. Similarly Kent *et al.* (1995) reported amplification of IS986 in a number species of environmental mycobacteria which contain insertion sequences belonging to the IS3 family, such as IS1141 in *M. intracellulare* and IS1137 *M. smegmatis*, hence raising doubts about the suitability of IS986 for the diagnosis of *M. tuberculosis*.

Further doubts on the use of IS986 have been raised recently by the findings of six strains of *M. tuberculosis* lacking this insertion sequence (van Soolingen *et al.*, 1993; Thierry *et al.*, 1995). Four of these strains were isolated from Vietnamese patients and one each from India and Burkina-Faso.

Since all the strains of *M. tuberculosis* complex analysed in the present study and those examined during validation of this technique (Chapter 4) produced IS986 positive results in a similar manner to numerous other published reports (Table 1.4; Wilson *et al.*, 1993; Sinclair *et al.*, 1995; del Potillo *et al.*, 1996; Weil *et al.*, 1996) it can be concluded that IS986 PCR used singly or in a multiplex PCR was 100% specific.

The *mtp40* PCR was described originally to be present in *M. tuberculosis* and absent in all *M. bovis* (Parra *et al.*, 1991; del Portillo *et al.*, 1991), hence a combined PCR with IS986 was able to differentiate *M. tuberculosis* from *M. bovis*. This was the basis of the multiplex PCR designed by Sinclair *et al.* (1995), which triggered a number of workers to develop variants of this protocol which could differentiate the two species. Liebana *et al.* (1996) developed a combined IS1081/*mtp40* PCR and found their protocol able to differentiate *M. bovis* from *M. tuberculosis*. Another

group of investigators (del Portillo *et al.*, 1996) developed a multiprimer PCR which utilised three sets of primers; 32kDa, IS6110 and *mtp40*; they also were able to differentiate *M. tuberculosis* from *M. bovis*. The above mentioned PCRs utilised the same primer sets for *mtp40* as those used in the current study, but primers for IS6110 were amplifying different sequences of this element. The multiplex PCR developed by Weil *et al.* (1996) was designed to amplify IS6110 and *mtp40*; the primer sequences for both target elements were unique to their study. In the initial work of the present study using *mtp40* PCR on strains of the *M. tuberculosis* complex from various countries (Chapter 4), all strains of *M. tuberculosis* tested were positive and *M. bovis* negative. All those strains were also subjected to a multiplex PCR developed by our group (Sinclair *et al.*, 1995) and identical results obtained IS986 and *mtp40*. In the present exercise, surprising results were encountered among the strains from Tanzania. Nine of 37 (24.3%) strains of *M. tuberculosis*, including the only strain from cattle, were found to lack the *mtp40* gene. These results were reproduced on more than two occasions (data not shown) and their biochemical characterisation was repeated by examining other sets of slopes of the original culture. Their identity on the basis of the biochemical tests was reconfirmed by SMRL. As Table 6.3 shows, all strains lacking *mtp40* originated from the Usangu Plains, representing 41% of the 22 strains of *M. tuberculosis* from this region. These results are in agreement with those recently published by Liebana *et al.* (1996) and Weil *et al.* (1996) who found 4/91 (4.4%) and (24%) 23/96 of the *M. tuberculosis* isolates they examined, respectively, did not contain the *mtp40* gene. These findings indicate that the *mtp40* gene is not found in all *M. tuberculosis* isolates and its potential in differentiating these species needs to be re-examined. Furthermore, the presence of this gene has been demonstrated in *M. africanum* (Liebana *et al.*, 1996) and *M. bovis* (van Embden *et al.*, 1995; Romano *et al.*, 1995; Weil *et al.*, 1996); these revelations cast further doubts on the specificity of this gene for *M. tuberculosis* only. In agreement with Weil *et al.* (1996), the current study also found that clusters of strains lacking *mtp40* had similar IS986 RFLP types. For example strains M23, M24 and M25 had RFLP type 12B; strains M29 and M30 had IS986 RFLP type 13E; strains M32 and M33 had RFLP type 15A (Table 6.3b).

As indicated above, the spoligotyping technique has the ability to distinguish *M. tuberculosis* from *M. bovis* on the basis of positive hybridisation with the last 5 of 43 spacers on the filter. In the present study, all strains that lacked *mtp40*, except one strain M35 had a spoligotype pattern characteristic of *M. tuberculosis*. Furthermore, strain M35 had a single copy of IS986, which raises concerns about its identity or a strain having features between *M. bovis* and *M. tuberculosis*.

The finding that the multiplex PCR may not be able to differentiate some strains of *M. tuberculosis* from *M. bovis* prompted the inclusion in this study of a reported *M. bovis* specific PCR (Rodriguez *et al.*, 1995). This putative specific DNA element for the PCR was obtained in a similar manner to that used in Chapter 5. The authors were able to clone a 500bp fragment from *M. bovis* and design a PCR to amplify that sequence. In their exercise, they tested a total of 20 *M. tuberculosis* and 11 *M. bovis* from their collection (including *M. bovis* ATCC 19210, *M. bovis* BCG Pasteur ATCC 27291, *M. tuberculosis* H37R_v, TMC102 and *M. tuberculosis* H37R_a ATCC25177), and found that there was no amplifiable product from *M. tuberculosis*. In contrast, the present study, yielded conflicting results. 37 of the 39 strains of *M. tuberculosis* subjected to this PCR were positive, which casts suspicion on the validity of the PCR as described by Rodriguez *et al.* (1995). Although one might raise concerns of false- positive results, this work used the same template DNA as that used for spoligotyping, which generated polymorphism among the strains and was able to differentiate *M. bovis* from *M. tuberculosis* (Figure 6.8).

The article by Rodriguez and co-workers (1995) indicated that when a 500bp DNA sequence was used as a probe on Southern blots of *EcoRI*- digested genomic DNAs, there were four similar size bands hybridised in both *M. tuberculosis* and *M. bovis* and a single 2.9Kb fragment found only in *M. bovis*. These workers admitted that that the results they obtained could be due to high degree of homology between the two species. It is likely that the presence or absence of the DNA fragment is variable from strain to strain and possibly the strains of *M. tuberculosis* they tested had a high degree of clonal relationship. In this study, the two strains of *M. tuberculosis* (M4 & M5) found to be negative in the *M. bovis* PCR were isolated from patients residing in Arusha. They had the same IS986 RFLP type (13A) and spoligotype (T), as another

two strains of *M. tuberculosis* which were positive by *M. bovis* PCR and which were isolated from patients in Arusha. The four patients attended different hospitals and their specimens were collected and cultured 4 months apart, confirming the suggestion that the absence of this DNA element might be confined to certain clones rather than all strains of *M. tuberculosis* as suggested by Rodriguez *et al.* (1995).

6.4.2. MOLECULAR TYPING

The epidemiology of bovine tuberculosis in Tanzania has not been elucidated fully up to the present time. Data collected during a survey carried out some four years ago, revealed the existence of an above average proportion of cases of extrapulmonary tuberculosis per total tuberculosis cases in regions with a high ratio of cattle to man (Catley 1992; Kazwala *et al.*, 1993). It was those figures which prompted the present study to be undertaken by collecting a number of specimens from human cases of tuberculosis as well as from cattle slaughtered in the study area. These materials were subjected to bacteriological analysis. Results presented in Chapter 3 showed the existence of the *M. tuberculosis* complex organisms in both cattle and human populations. In order to determine the similarity of strains from the two sources, molecular typing techniques, namely RFLP and spoligotyping, were used to determine the genetic profile of the strains involved. A polymorphic G-C rich element, pTBN12 was used as a probe to type strains of *M. bovis*. Previous studies have shown that the vast majority of *M. bovis* strains contain a single IS986 element, often at the same chromosomal locus, thus hampering strain differentiation with IS986 as a probe (Chapter 4). Strains of *M. tuberculosis* were typed using the standard procedure as described (van Embden *et al.*, 1993). In order to obtain a consensus in the typing of the two species of mycobacteria, all the strains were also typed using a spoligotyping technique.

The results of pTBN12 typing of *M. bovis* from cattle and man has shown a rather heterogeneous population of this species spread all over Tanzania, assuming that the present sample is representative. There were 13 different pTBN12 RFLP types encountered. It has been put forward that where tuberculosis is endemic, then the

number of strain types circulating in a population would be very low (Hermans *et al.*, 1995; van Soolingen *et al.*, 1995). This feature was reproduced in the current study. Twenty strains of *M. bovis* were isolated from cattle and man from Arusha and 12 more than half (55%) were of the same type (pTBN12 RFLP type C). Similarly 42% of the strains recovered from the Usangu Plains belonged to the same pTBN12 RFLP pattern (A).

The genetic relatedness between the pTBN12 RFLP patterns (Table 6.5a) indicated a high degree of relatedness (86%) between the dominant pTBN12 genotypes existing in Tanzania i.e. pTBN12 RFLP types A and C, which indicates that these strains might have evolved from the same ancestor recently. The lowest genetic relatedness value (29%) was that between pTBN12 RFLP types F and M. These two genotypes were found in two strains recovered from a bovine lymph node and a milk sample from the Usangu Plains. The diversity of their DNA profiles also was confirmed by IS986 RFLP which revealed that pTBN12 RFLP type F had 6 copies of IS986, while the strain with pTBN12 RFLP type M had only one copy of IS986. In contrast to the diversity revealed by RFLP, their genetic relatedness according to spoligotyping was surprisingly high (79%). This observation is in agreement with that put forward by van Soolingen and co-workers (1993), who found that strains with a single copy of IS986 were not necessarily of the same genotype when other genetic markers such, as DR or PGRS were used as probes.

There was an overlap of pTBN12 RFLP type A between strains recovered from Arusha and the Usangu plains and the only *M. bovis* recovered from a human case in Dar-es-Salaam had a DNA profile similar to that found only in strains recovered from cattle in Arusha (pTBN12 RFLP type C). This observation confirmed further the suggestion put forward by Crawford (1993) and Hermans *et al.* (1995) suggesting that the variability of isolates may be greater in areas that have a low incidence of tuberculosis than in areas where tuberculosis is epidemic. This suggestion would only be true if each of the two zones in the current study i.e. Arusha representing the Northern Zone and Usangu Plains representing the Southern Zone of Tanzania, are considered individually. The diversity of the RFLP and spoligotype patterns observed in Tanzania probably reflects the extensive internal movements of cattle belonging to

pastoralists and, as a consequence of that, the spread of different strains of *M. bovis* throughout the country. The single isolate of *M. bovis* from a human case in Dar-es-Salaam which had a similar pTBN12 RFLP to that found in cattle in Arusha, is further evidence of spread of the disease in various parts of the country.

Despite the isolation of few isolates of *M. bovis* from human cases of tuberculosis, some of these strains were genetically similar to those found in cattle. The two strains of *M. bovis* from man, which showed the same pTBN12 RFLP pattern, were however, found not to resemble each other when different probes or typing techniques were used. This finding, and that discussed above, have shown further that target DNA elements (i.e. pTBN12, IS986 & DR spacers) for typing are not genetically linked and underline the need for standardised DNA fingerprinting not only for *M. tuberculosis* as described by van Embden *et al.* (1993), but also for strains of *M. bovis*. The latter activity is the focus of the work of the Standardisation of DNA Fingerprinting Sub-committee of the Animal Tuberculosis Section of the International Union Against Tuberculosis and Lung Diseases, of which the author is a member.

The comparison of two or three types of DNA fingerprinting (Tables 6.3a and b) has shown that different probes or typing techniques would, to a limited extent, produce the same fingerprint among the *M. bovis* strains. For example, most strains (80%) with pTBN12 RFLP type C also produced spoligotype L. Therefore, in order to make better use of the different typing techniques, the genotypes generated by the different probes/technique were combined to give an overall type (Tables 6.3a and b) which was able to differentiate further the strains of *M. bovis*. This approach, which was used also by Skuce *et al.*, (1994), enabled the assignation of 33 strains of *M. bovis* to 23 different DNA fingerprint types instead of 13 and 12 offered by pTBN12 RFLP and spoligotyping, respectively.

As described above, the use of spoligotyping in this study had two main objectives, namely, to type strains of *M. bovis* and *M. tuberculosis* and to differentiate strains of *M. tuberculosis* from strains of *M. bovis*. As regards typing of the 34 strains of *M. bovis*, spoligotyping did not offer much difference from pTBN12 in terms of the number of spoligotypes, which classified strains into 14 types vs 13 produced by

pTBN12 RFLP. However, sixteen strains (47%) were grouped into one spoligotype, indicating its relatively lower power to discriminate strains of *M. bovis*. Despite this shortcoming this PCR based technique proved to be of great potential in typing strains of the *M. tuberculosis* complex as it was very fast, easy to perform and did not require such high quantity and quality of DNA as Southern blotting.

Regarding differentiation of strains of *M. bovis* from *M. tuberculosis*, there were peculiar spoligotypes exhibited by three of the five *M. bovis* strains recovered from human cases of tuberculosis. Strains M27 (Type AA), M33 (Type T) and M11 (Type U) gave hybridization with spacers 39 through 43. According to initial work by Kamerbeek *et al.* (1993) as communicated to our group, these patterns should have been regarded as *M. tuberculosis*. A recent communication by Dr. J.D.A van Embden to a conference on tuberculosis in animals in Dunedin, New Zealand (1995) indicated that some strains of *M. bovis* might hybridise the last five contiguous spacer located near the 3' end of DR.

The isolation of *M. tuberculosis* from cattle tissues has been cited but only rarely such as Lesslie (1960) in U.K. and Waddington (1965) in Kenya. In the present study a single strain (C22) of *M. tuberculosis* apparently was isolated from cattle tissues. This strain was found to be Spoligotype M whose genetic relatedness to the major spoligotypes found in cattle (L and E) was between 65-74%.

In 1993, international consensus was reached on a standardized method of *M. tuberculosis* RFLP analysis (van Embden *et al.*, 1993), and this technique has recieved wide usage all over the world (Table 6.1). The success of this technique in typing strains of *M. tuberculosis* led to it use in typing *M. bovis* (Table 6.2). In contrast to *M. tuberculosis*, typing of *M. bovis* with this technique has been less successful (Skuce *et al.*, 1994; van Soolingen *et al.*, 1994; Szewzyk *et al.*, 1995; Perumaala *et al.*, 1996). Although all strains of *M. bovis* encountered in the present study were RFLP typed using a pTBN12 probe, the findings of van Soolingen *et al.* (1994) showed that some strains of *M. bovis* from man and from zoo and wild animals had multiple (>6) copies of IS986. This finding prompted the current study to probe some of the blots, which had been probed earlier with pTBN12, with IS986. Eighteen strains of *M. bovis*, including 4 from man, were typed using the IS986

probe, of which only two had a single band at 1.9 and 4.5Kb while 14 strains produced five or more bands. The majority of *M. bovis* isolates from cattle contained five to six copies of IS986. This finding is in agreement with a recent report by Perumaala *et al.* (1996) who found some *M. bovis* isolates in U.S.A. that originated from cattle and deer, harboured multiple copies of IS986. Two of the four human isolates were found to contain 13 and 15 copies of IS986 and the banding pattern (15A), in one of the strains, was similar to that amongst *M. tuberculosis* found in the same geographical area. High copy numbers of IS986 amongst *M. bovis* recovered from human cases also has been recorded in the Netherlands (van Soolingen *et al.*, 1994).

As the study involved isolation of mycobacteria from human clinical specimens, there were numerous isolates of *M. tuberculosis* recovered from cases of tuberculosis amongst the pastoralists. These strains were subjected to molecular identification and typing in the same manner as *M. bovis*. Results of the present study (Tables 6.3b and Figure 6.4) indicate that strains with 13 copies of IS986 i.e. RFLP type 13A and its variants were the most prevalent in Arusha, whereas strains from the Usangu Plains had one to 15 copies of IS986, with most of the isolates (77.3%) having more than 10 copies of IS986.

In studies carried out in Ethiopia and Tunisia (Hermans *et al.*, 1995) and in East Asia (van Soolingen *et al.*, 1995) these workers indicated that in countries with a high incidence of tuberculosis there are region-specific *M. tuberculosis* types such as the "Beijing family". A similar feature was revealed in Tanzania by the current study. Although the number of strains examined was much lower than in the studies described above, there was a dominance of particular IS986 RFLP types in regions/zones. For example, in the present study, none of the strains found in the Usangu Plains had an IS986 RFLP type similar to those found in the northern part of the country, indicating that there are clones of *M. tuberculosis* which are only found in a particular geographical area.

Dar-es-Salaam is a very cosmopolitan city and its main hospital (Muhimbili Medical Centre) is the national referral centre for many disease conditions including tuberculosis, therefore, the diversity of IS986 RFLP patterns exhibited by strains of

M. tuberculosis from this region would be a reflection of the polymorphism of strains circulating in various parts of Tanzania.

In a study of tuberculosis in 134 cases (68 with and 66 without HIV) in Dar-es-Salaam by Yang *et al.* (1995), these workers observed a large number of unique patterns. However, about 40% of the strains examined were classified into 3 big clusters, each of which was very similar to the other. Apparently, that study failed to show a clonal relationship among the strains recovered from cases with and without HIV (Yang *et al.*, 1995). In the current study, strains were clustered on the basis of the number of copies of IS986 and majority of the strains (64.5%) were those which belonged to Cluster III (strains with > 10 copies of IS986) while the cluster of low copy number (1-5) isolates formed 16.1%. This distribution between clusters is comparable to that found by the above mentioned study, but different from a Tunisian study (Chevrel-Dellagi *et al.*, 1993). Strains with low copy number were mostly found in the Usangu Plains, which indicated that relatedness of DNA fingerprint pattern tended to be in accordance with the geographical origin of the strains as has been observed among strains, encountered in Malawi and Kenya (Godfrey-Faussett and Stoker 1992) and in Greenland (Yang *et al.*, 1994).

As regards spoligotyping, 34 strains of *M. tuberculosis* analysed in the current study revealed 20 different spoligotypes, with spoligotype T being dominant (35.3% of strains). This spoligotype was found in all but two of the strains that had 13 to 15 copies of IS986. One *M. bovis* from a human case, which had fifteen copies of IS986, also had this spoligotype. However, there were four strains of *M. tuberculosis* from the Usangu Plains which had the same IS986 RFLP type (12B) but had four different spoligotypes (R, S, V & Z). Therefore, spoligotyping as applied in the current study was not able to restrict spoligotypes to particular IS986 RFLP patterns or geographical regions. This is in contrast to a study on strains of *M. tuberculosis* from East Asia (van Soolingen *et al.*, 1995), in which one spoligotype was common to all the strains of the so called Beijing family, as well as strains from Mongolia and other countries, including some Dutch strains which had a unique IS986 RFLP type.

In view of the above findings it can be concluded that the existing molecular techniques are able to correctly identify strains of the *M. tuberculosis* complex from Tanzania, but were not able to fully distinguish the closely related species within the *M. tuberculosis* complex (i.e. *M. bovis* from *M. tuberculosis*) as identified by biochemical means. The molecular typing techniques were able to assign these species to various genotypes which showed a region or zone specificity. Importantly, these techniques were able to show that *M. bovis* strains from human cases of tuberculosis were related to those found in cattle in Tanzania, emphasizing the zoonotic importance of the *M. bovis* infection in Tanzania. Although some of the *M. bovis* strains from man did not produce DNA fingerprints matching those found in cattle, these isolates were found to be 70 to 80% genetically related to the dominant DNA fingerprints of isolates from cattle. The close resemblance of these genotypes indicates that these strains might have evolved from a common ancestor in the recent past.

The evidence of overlap between DNA fingerprints of *M. bovis* from cattle and man has once more highlighted a need for a veterinary and medical approach in the control of tuberculosis in Tanzania and probably in other developing countries.

CHAPTER 7

**THE DEVELOPMENT OF A MODIFIED LIQUID
MEDIUM FOR THE GROWTH OF
MYCOBACTERIUM BOVIS FOR MOLECULAR
METHODS.**

CHAPTER 7 THE DEVELOPMENT OF A MODIFIED LIQUID MEDIUM FOR THE GROWTH OF *MYCOBACTERIUM BOVIS* FOR MOLECULAR METHODS.

7.1. INTRODUCTION

Cultivation of strains of *M. tuberculosis* complex organisms has been achieved by the use of solid media; such as Middlebrook 7H10 (Middlebrook and Cohn 1958), Loewenstein-Jensen (L-J) and Stonebrink (Jensen 1955; Stonebrink 1958) and blood based media -B83 (Cousins *et al.*, 1989) or a liquid medium such as Middlebrook 7H9 (Middlebrook and Cohn 1958). In most of the media intended for isolation of *M. tuberculosis*, glycerol has been added as a source of carbohydrate and thereby producing eugonic growth (Boissevain 1943a&b; Stonebrink 1958). The addition of glycerol was found to impair the growth of the bovine type bacilli, which would either grow very slowly or not at all (Boissevain 1943b). However, Grange *et al.* (1990) have reported that 11% of 150 *M. bovis* isolated from cattle and other animals in Ireland grew well on glycerol and/or pyruvate containing medium.

According to Stonebrink (1958), the lack or poor growth of bovine type bacilli on media containing glycerol arose from the dehydrogenation system of this species. *M. bovis* is known to lack the appropriate dehydrogenase enzyme which could break glycerol into lower carbohydrates, which includes pyruvic acid. Glycerol has been used commonly in media when large crops of tubercle bacilli have been required. This compound greatly stimulates oxygen consumption of *M. tuberculosis* and therefore enhances its growth (Wayne and Kubica 1986). It was Boissevain (1943a) who first replaced glycerol with pyruvate and found that the growth of bovine type

bacilli was very much enhanced, while its addition in medium intended for isolation of human type bacilli had no effect on the growth.

The commonly used media for the isolation of *M. bovis* have been Stonebrink medium (Stonebrink 1958), L-J supplemented with pyruvic acid (Jensen 1955), blood based agar B83 (Cousins *et al.*, 1989) and modified Middlebrook 7H11 agar medium (Gallagher and Horwill 1977). All these media incorporate pyruvic acid or sodium pyruvate and are able to produce colonies on their surface between four to eight weeks.

Studies on the molecular epidemiology of *M. bovis* (Table 6.2) have utilised techniques such as restriction enzyme analysis (REA), restriction fragment length polymorphism (RFLP) and pulsed field gel electrophoresis (PFGE). These techniques require at least 1-2µg of good quality DNA, which can only be extracted from at least 100mg of wet weight of mycobacterial cells (Whipple *et al.*, 1987). This amount of cells is not easily achieved with the commonly used cultivation techniques for the following reasons: i) the long doubling time of the organism on the above mentioned media (16-18hrs) and ii) scant amounts of the cells produced on these media. In order to get around the latter shortcoming, several researchers have utilised up to 10 plates or slants (Cousins *et al.*, 1993b; Feizabald *et al.*, 1996).

Harvesting of cells on solid media requires scraping cells from the agar surface and suspending them in a liquid prior to heat inactivation (Cousins *et al.*, 1993b). This protocol is hazardous as there is possibility of aerosol generation. Some laboratories therefore have resorted to the use of liquid medium, which would require not opening vials until the cells have been heat killed and the cell pelleted by centrifugation within the same container (van Soolingen *et al.*, 1991; Skuce *et al.*, 1994; Perumaalla *et al.*, 1996). The commonest liquid medium is Middlebrook 7H9 broth (Middlebrook and Cohn 1958). This medium is specifically intended for the isolation of *M. tuberculosis* and requires the inclusion of albumin dextrose enrichment supplement (ADC).

In studies described in Chapters 4 through 6, strains of *M. bovis* as well as *M. tuberculosis* were subcultured onto slopes of L-J medium with and without sodium pyruvate in order to produce at least 100mg of wet weight of mycobacterial cells for

DNA extraction. This activity was found to be cumbersome as sometimes particles of the friable egg medium also were included, giving rise to false wet weights, which in turn resulted in lower yields of extracted DNA per unit weight. For these reasons and those mentioned above it was decided to utilise Middlebrook 7H9 liquid medium. This medium also was found not to yield enough cells for DNA extraction, hence up to four 10ml vials were used to grow the cells. This resulted in using enormous amounts of medium, making this exercise less cost-effective. Furthermore, the incubation period before harvesting the cells was at least four to six week.

7.1.1. AIMS OF THE STUDY

As pyruvic acid is essential for the growth of *M. bovis* in several media, the present study investigated the potential of this supplement to improve growth of *M. bovis* in Middlebrook 7H9 liquid medium. For comparison purposes *M. tuberculosis* strains also were inoculated into the modified medium. The study compared the growth of the two species in Middlebrook 7H9 broth with and without addition of sodium pyruvate. This exercise was carried out in two phases. The first determined the appropriate time for growth and harvesting of an appropriate wet weight of cells for molecular methods (100mg) on this modified medium, and the second determined which volume of modified medium would yield 100mg of wet weight of the cells at the log phase of growth curve established by phase one of this study.

7.2. MATERIALS AND METHODS

7.2.1. MYCOBACTERIUM STRAINS

Ten strains each of *M. bovis* and *M. tuberculosis* from the field study conducted in Tanzania, which have been the subject of analysis in various chapters of this work (Chapters 3 through 6) were used. All the strains were sub-cultured onto L-J medium

with pyruvate prior to use in this study. All the strains were coded, EB1 to EB10 for *M. bovis*, while those of *M. tuberculosis* were coded ET1 to ET10. The reason for coding was for simplicity of recording.

7.2.2. MEDIA PREPARATION

Middlebrook 7H9 medium [composition per 1 litre; ammonium sulphate 0.5g, L-glutamic acid 0.5g, sodium citrate 0.1g, pyridoxine 0.001g, biotin 0.0005g, disodium phosphate 2.5g, monopotassium phosphate 1g, ferric ammonium citrate 0.04g, magnesium sulphate 0.05g, calcium chloride 0.0005g, zinc sulphate 0.001g and copper sulphate 0.001g] was prepared according to the manufacturer's instructions (DIFCO Laboratories, Detroit, Michigan, USA); simply, 4.7g of the commercially available Middlebrook 7H9 powder were dissolved in 900ml of distilled water. For the broth that incorporated sodium pyruvate, 4g of this compound were added after the 7H9 powder had been dissolved completely. The media were autoclaved for 10 minutes at 15 pounds pressure at 121°C and after cooling to about 45°C, the albumin-dextrose-catalase enrichment supplement [(ADC) - 100 ml contained bovine albumin fraction V 5g, dextrose 2g, and beef catalase 0.003g] supplements (DIFCO) was aseptically added to a final concentration of 10%. 10ml volumes of the media were then dispensed aseptically into sterile 25ml glass universals and stored at room temperature until used.

A modified Middlebrook 7H11+ medium was used to determine viable counts in inocula and to grow cells. One litre of this medium was made up as follows; 21g of Middlebrook 7H11 agar [composition per 1 litre; ammonium sulphate 0.5g, L-glutamic acid 0.5g, sodium citrate 0.4g, pyridoxine 0.001g, biotin 0.0005g, disodium phosphate 1.5g, monopotassium phosphate 1.5g, ferric ammonium citrate 0.04g, magnesium sulphate 0.05g, malachite green 0.001g, pancreatic digest of casein 1g and Bacto agar 15g - (DIFCO)] and L- asparagine 0.3g. The mixture was autoclaved as above, cooled to 45°C, then supplement with 10% OADC [Oleic acid 0.05g, bovine albumin fraction V 5g, dextrose 2g, and catalase beef 0.004g, sodium chloride

0.85g and water to make 100ml volume (DIFCO)], 200ml heat inactivated new born calf serum (GIBCO) 200ml and 2 mycobacteria selectatabs.

7.2.3. EXPERIMENTAL DESIGN

7.2.3.1. Experiment 1. Evaluation of the effect of sodium pyruvate supplementation on growth of *M. bovis* and *M. tuberculosis* on 7H9 liquid medium.

7.2.3.1.1. Viable counts

All the strains mentioned above were grown at 37°C on L-J medium with pyruvate for four weeks and harvested by scraping. A loopful of cells was suspended in phosphate buffered saline and washed thrice. The washed suspension resuspended and adjusted with Middlebrook 7H9 broth without ADC supplement to around 10^7 to 10^8 cfu/ml using MacFarland's standards.

For each test strain, duplicate 9.9 ml broths of Middlebrook 7H9 with and without sodium pyruvate were inoculated with 10µl of the appropriate inoculum. The broths were then incubated for 4 weeks at 37°C.

Viable counts for all 20 test strains were determined at day 0 and at 28 days post-inoculation. The viable counts of two strains, one of *M. bovis* (EB1) and one of *M. tuberculosis* (ET1), were determined at weekly intervals up to the sixth week post inoculation.

Viable counts were determined using modified Middlebrook 7H11+agar without glycerol. A 0.01ml volume was spread evenly on the surface of the medium and incubated for 3-4 weeks or until growth appeared.

7.2.3.1.2. Wet weights

In order to assess the effect of sodium pyruvate supplementation on the yield of cells required for molecular methods. Batches of three strains each of *M. bovis* and *M. tuberculosis* were harvested at the end of the fourth through sixth week of

experiment, and the wet weights measured. The harvesting of cells was carried out as described in 2.2.1.3.

A comparison between viable counts and wet weight was made by determining correlation of coefficient (r) between the two variable by the method described below.

Part of the work in this trial was undertaken in collaboration with Dr. J.F. Porter and Mrs. K.M. Connor, Division of Microbiology, Moredun Research Institute, to whom I express my sincere thanks.

7.2.3.2. Experiment 2 - Determination of growth rate and appropriate culture volume of modified 7H9 broth to obtain 100mg wet weight growth of *M. bovis*.

This trial was conducted to extend the findings from Experiment 1.

In order to determine the rate of growth of *M. bovis*, cultures were monitored weekly and recorded by plus (+) or negative (-) signs as follows: -, no obvious growth; \pm , scant growth; +, appearance of cell clumps upon shaking; ++ obvious sedimentation of cell clumps at the bottom of the flask, and +++ pellicle formation.

Regarding the determination of volumes appropriate to obtain 100mg of wet weight, the following volumes were selected for this trial; 10, 20, 50 and 100ml. The amount of cells inoculated per ml of medium (10^5 cfu/ml) was the same as that used for inoculation in Experiment 1.

For cultivation of these volumes, tissue culture flasks (Corning Inc., Corning, N. York, USA) of the following sizes were used, 25, 75, 125, 150 cm² for 10, 20, 50 and 100 ml of the broth, respectively. For each volume, five flasks were used, and all the flasks for this experiment were inoculated with *M. bovis* strain EB1, which also was used in weekly, assessment of viable counts in Experiment 1. Growth conditions were the same as those described above and cells were harvested at the end of the third week of incubation.

7.2.4. STATISTICAL ANALYSIS

Comparison of the differences between the means of viable counts as well as the wet weights was carried out as described by Thrusfield (1986). In order to measure the association between wet weights and viable counts, the correlation of coefficient (r) was determined by the method described by Mead *et al.* (1993).

7.3. RESULTS

7.3.1. EXPERIMENT 1. EFFECT OF SODIUM PYRUVATE SUPPLEMENTATION OF 7H9 LIQUID MEDIUM ON VIABLE COUNTS OF *M. BOVIS* AND *M. TUBERCULOSIS*

The growth of *M. bovis* EB1 and *M. tuberculosis* ET1, monitored for a period of six weeks, showed that in broth containing pyruvate there was an initial lag phase during the first week, which was followed by a rise in the number of viable cells for up to three weeks and then steady growth (Figure 7.1). There was a significant difference between the viable counts taken at day 0 and at the end of experiment ($p < 0.03$). The growth of *M. bovis* strain EB1 in broth lacking pyruvate showed no significant increase ($p > 0.05$) in viable counts and in fact the viable count dropped slightly as the incubation continued (Figure 7.1). Comparison of the viable counts at end of experiment shows significantly higher viable counts were encountered in EB1 grown in liquid medium with pyruvate compared to medium without pyruvate ($p < 0.05$) (Figure 7.1). Although the growth of strain ET1 on medium containing pyruvate was significantly higher ($p < 0.05$) than in medium without pyruvate at weeks two to four post-inoculation, there was, however no significant difference ($p > 0.05$) of viable counts at the end of experiment (Figure 7.1). The log phase growth of *M. bovis* grown in pyruvate containing medium achieved its peak at week three and a constant growth was observed thereafter, while *M. tuberculosis* seemed to continue growing exponential through the study (Figure 7.1). Figure 7.2 shows that broths with

pyruvate had copious amounts of cell clumps at the bottom compared to the scant amount of cell clumps found in medium without pyruvate at week four of growth.

When the viable counts of the rest of the strains was performed at week four, the mean counts were comparable to those found for strains EB1 and ET1 (Table 7.1). The mean viable counts produced by strains of *M. bovis* cultivated in Middlebrook 7H9 with pyruvate added was significantly higher than that produced by the same strains in broth without pyruvate ($p < 0.0001$) (Table 7.1 and Figure 7.2). As regards strains of *M. tuberculosis*, there also was a significant difference in viable counts encountered when these strains were grown in broth with and without pyruvate ($p < 0.01$) (Table 7.1 and Figure 7.2).

The average wet weights produced by batches of 3 different strains of *M. bovis* harvested at weeks 4, 5 and 6 post-inoculation from broths with pyruvate supplement were found to be significantly different ($p < 0.05$) from the wet weights in the same strains grown in broths without supplementation (Figure 7.3). There was no significant increase ($p > 0.05$) in the wet weights of *M. tuberculosis* cells grown in Middlebrook 7H9 broth supplemented with pyruvate harvested between weeks 4 to 6 (Figure 7.3).

A comparison of the mean viable counts and mean wet weight obtained from both broths (Figure 7.4) revealed a positive correlation between the two parameters ($r = 0.85$).

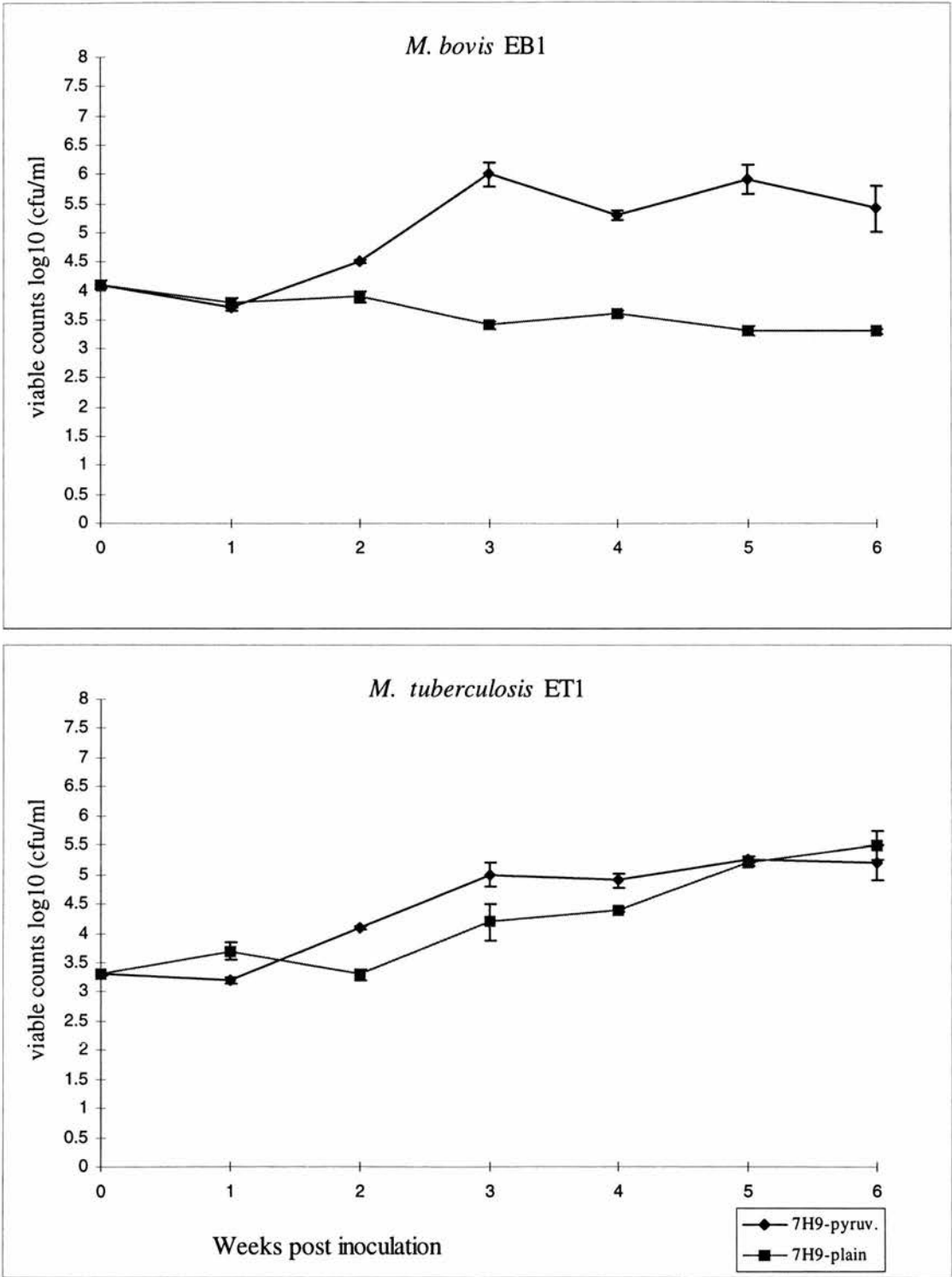


Figure 7.1. The comparison of viable counts produced by *M. bovis* (EB1) and *M. tuberculosis* (ET1) on Middlebrook 7H9 liquid medium with (7H9-pyruv.) or without (7H9-plain) pyruvate.

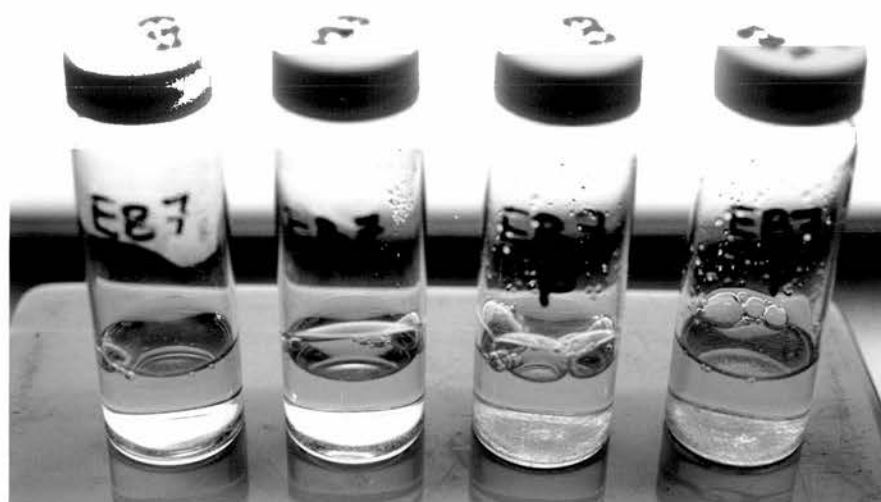


Figure 7.2. Comparison of growth of *M. bovis* and *M. tuberculosis* in Middlebrook 7H9 broth with and without pyruvate at week 4 post inoculation. The bottles with medium containing pyruvate are marked with a P, and show accumulation of clumps of cells at the bottom.

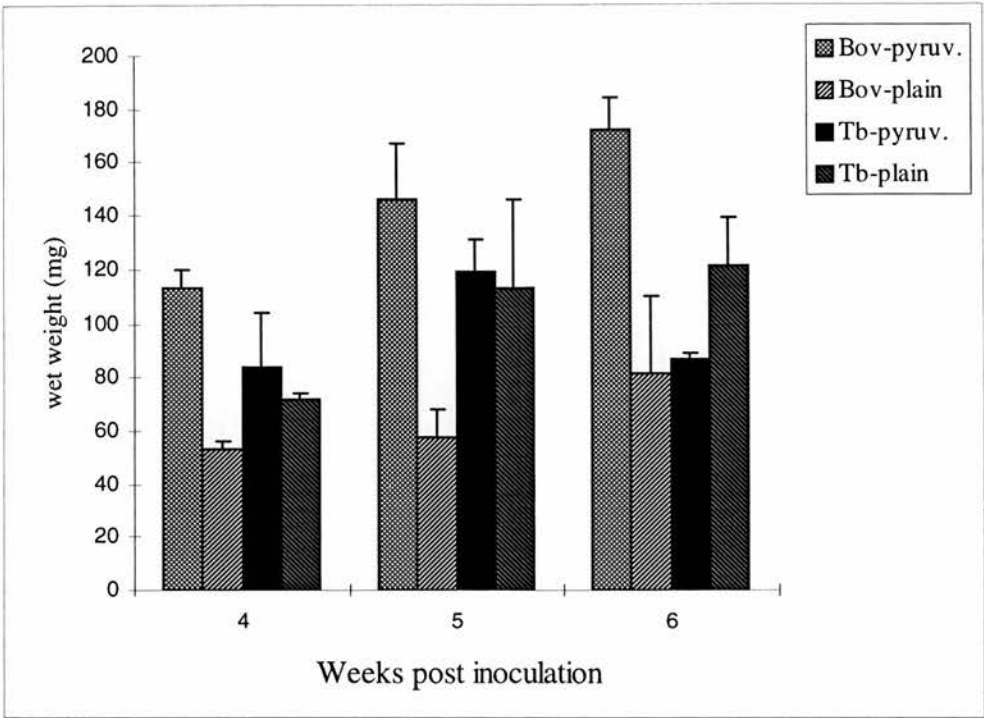


Figure 7.3. The comparison of the mean wet weights (mg per 20ml) produced by batches of three strains of *M. bovis* and *M. tuberculosis* grown on Middlebrook 7H9 broth with (Bov-pyruv.; Tb-pyruv.) and without (Bov-plain; Tb-plain) pyruvate added, harvested at weekly intervals between weeks 4 and 6.

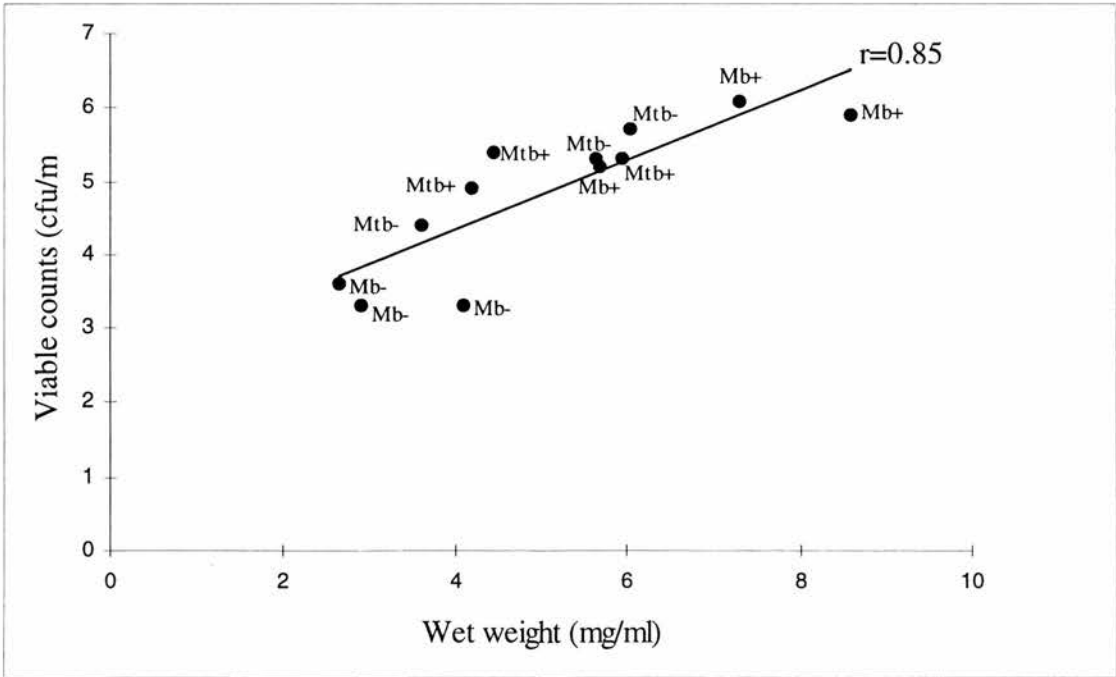


Figure 7.4. Comparison of mean viable counts and mean wet weight of the strains of *M. bovis* (Mb) and *M. tuberculosis* (Mtb) harvested at the end of weeks 4, 5 and 6. (r = correlation of coefficient; plus or minus sign indicate medium with or without pyruvate added).

Table 7.1. The mean viable counts at the end of week four post inoculation

<i>M. bovis</i>			<i>M. tuberculosis</i>		
Strain No.	7H9+pyruvate	7H9 plain	Strain No.	7H9+pyruvate	7H9 plain
EB1	5.3±0.08*	3.6±0.05	ET1	4.9±0.13	4.4±0.03
EB2	4.6±0.10	3.8±0.02	ET2	4.2±0.35	3.4±0.01
EB3	4.4±0.26	3.2±0.07	ET4	5.0±0.12	4.5±0.09
EB5	5.0±0.10	3.8±0.03	ET5	4.8±0.10	4.0±0.08
EB6	4.3±0.31	4.2±0.08	ET6	4.5±0.12	4.4±0.09
EB7	4.8±0.04	3.7±0.06	ET7	5.1±0.22	3.8±0.08
EB8	4.9±0.04	3.5±0.02	ET8	3.5±0.14	3.7±0.12
EB9	4.8±0.08	3.7±0.10	ET9	4.8±0.06	4.4±0.14
EB10	4.3±0.03	3.4±0.03	ET10	5.1±0.13	4.2±0.12
mean±se	4.7±0.12	3.7±0.09	mean	4.7±0.17	4.1±0.13
Student T test = p<0.0001			Student T test = p<0.01		

*mean counts in log₁₀ cfu/ml se = standard error of mean

7.3.2. EXPERIMENT 2. DETERMINATION OF RATE OF GROWTH AND APPROPRIATE VOLUME FOR GROWTH OF *M. BOVIS* IN MODIFIED MIDDLEBROOK 7H9 BROTH.

Visible growth started to appear as early as seven days post-inoculation in flasks with 10 and 20ml volumes and copious amounts of clumps and pellicle formations were seen at the end of the second week (Table 7.2). Growth in flasks containing 50 and 100ml was not visible until the end of second week and had not formed pellicles by the end of the experiment (Week 3; Table 7.2).

The flasks containing different volumes of broth yielded various weights, as indicated in Figure 7.5. It can be seen that, proportionally, the lower volumes i.e. 10 and 20ml, gave a higher number of cells per ml (6.4 - 7.1 mg/ml) compared to higher volumes

(4.7 and 5.3 mg/ml for 50 and 100ml respectively). This difference was statistically significance ($p<0.05$)

Table 7.2. The rate of growth of *M. bovis* on different volumes of the modified Middlebrook 7H9 broth.

Volume of medium (ml)	Duration of incubation weeks post inoculation		
	1	2	3
10	+	+++	+++
20	+	++	+++
50	±	+	++
100	-	+	++

* scores given as described in 7.2.2

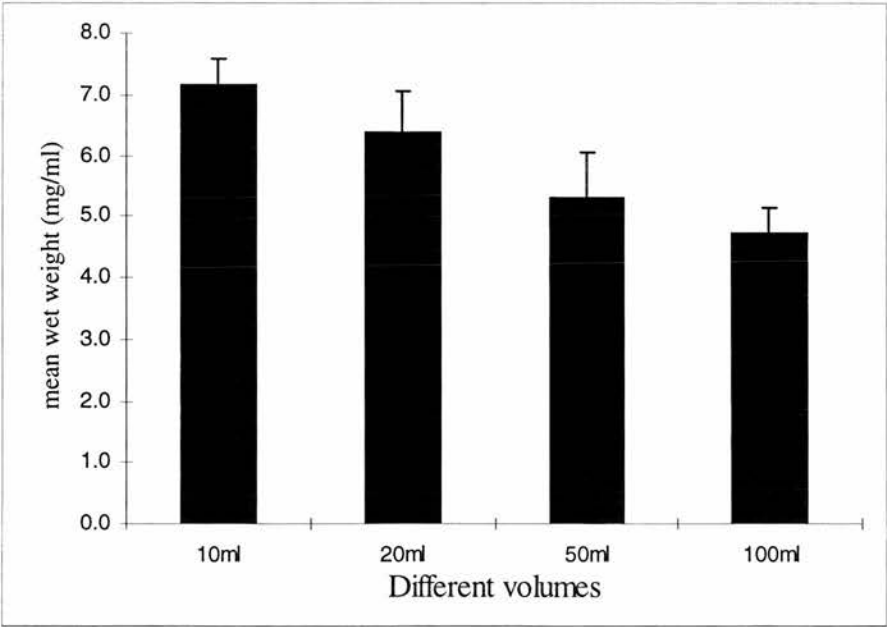


Figure 7.5. The wet weight (mg/ml) yields obtained from cultivation of *M. bovis* (EB1) in different volumes of modified Middlebrook 7H9 broth.

7.4. DISCUSSION

In the present study it has been demonstrated that the supplementation of Middlebrook 7H9 liquid medium with sodium pyruvate enhanced the growth of *M. bovis* which resulted in better yields of cells for molecular methods in the most cost-effective manner compared to unsupplemented medium.

Credit for the development of media containing pyruvate to improve the for isolation of *M. bovis* should be accorded to Boissevain (1943a&b). This scientist was the first to recognise that the growth of strains of *M. bovis*, then known as bovine tubercle bacillus was greatly impaired by the inclusion of glycerol in growth medium. Boissevain (1943b) substituted glycerol with pyruvic acid, which resulted in vigorous growth of the dysgonic bacilli of the bovine type.

Glycerol is an important component of media intended for isolation of human types of tubercle bacilli, especially when large crops of bacilli are desired (Wayne and Kubica 1986). *M. tuberculosis* grows happily in any media containing glycerol as its dehydrogenase system is able to break down glycerol into simpler carbohydrate compounds (Stonebrink 1958). Since this feature is lacking in strains of *M. bovis*, growth of this species is severely affected by this compound (Stonebrink 1958). Pyruvic acid is one of the derivatives of the breakdown of glycerol. It does not need to be dehydrogenated and can be degraded further by catalase, a common enzyme in many mycobacterial species (Stonebrink 1958).

In the present study, 0.4% sodium pyruvate was added to Middlebrook 7H9 liquid medium for the purpose of producing large amounts of bovine tubercle bacilli for molecular methods. The results showed significant differences in both the viable counts of four weeks cultures ($p < 0.0001$), as well as the wet weights of cells harvested at the end of week 4,5, and 6 ($p < 0.001$ to 0.01). The inclusion of this substance in the Middlebrook 7H9 liquid medium was more beneficial to the growth of *M. bovis* than *M. tuberculosis* which from week five to six seemed to grow faster in medium without pyruvate (Figure 7.1 and 7.3). The lack of difference in the wet weight of *M. tuberculosis* could attributed to the fact that Middlebrook 7H9 medium was intended originally for cultivation of *M. tuberculosis* (DIFCO Manual No. 1458 -

February 1980) hence the available nutrients may favour the growth this species, even without addition of pyruvate.

Cells of mycobacteria are highly hydrophobic and that is why they form clumps. This has resulted to some workers insisting on the use of detergents, such as Tween-80, in liquid medium when accurate measures of growth dynamics by means of a spectrophotometer are needed (Wayne 1994). In the current study, detergent was not incorporated in the medium as the ultimate goal was to demonstrate the effect of sodium pyruvate on yields of cells (regardless of whether they were clumped or in individual cells) rather than growth dynamics. Therefore, cell suspensions for viable counts were taken from bottles after a thorough shaking, which created a homogenous suspension. Moreover, the comparison made between the mean wet weights and viable counts of the cells at weeks 4, 5, and 6 have shown a positive correlation ($r=0.85$) between the two parameters (Figure 7.4). The positive correlation between the wet weights and viable counts observed here is, therefore, in disagreement to the statement put forward by Wayne (1994), who indicated that unless a detergent is added into a liquid medium, it is useless to determine the growth of mycobacteria by a colony counting method.

The approach undertaken in the course of the current study, i.e. modifying a liquid medium intended for isolation of *M. tuberculosis*, also has been employed by a number of previous workers, who improved solid media originally intended for isolation of *M. tuberculosis*. For example, Jensen (1955) substituted the glycerol used in IUT medium with 0.4% sodium pyruvate creating modified L-J medium. Stonebrink (1958) incorporated 0.1% sodium pyruvate into a medium developed in 1951 for the isolation of bovine tubercle bacilli from its outset. Recently, Cousins *et al.* (1989) developed a blood based medium B83 specific for *M. bovis*. This medium was a modification of the blood medium for *M. tuberculosis* described by Tarshis (1953). In B83 medium, glycerol was replaced by sodium pyruvate and L-asparagine. Gallagher and Horwill (1977) described a selective oleic acid albumin agar medium for the cultivation of *M. bovis*. In this medium, which is modified Middlebrook 7H11 medium, fresh bovine serum and lysed red cells were added to encourage growth of *M. bovis*.

Comparative studies on the use some of these media and others was addressed by Lesslie (1959), Corner and Nicolacopoulos (1988) and Cousins *et al.* (1989). It was demonstrated that the media containing pyruvate were effective in the isolation of *M. bovis* from clinical material in a similar manner to L-J medium (Lesslie 1959; Corner and Nicolacopoulos 1988; Cousins *et al.*, 1989).

In a recent survey on the standardisation of methods for DNA fingerprinting of *M. bovis*, conducted by the working group of IUATLD committee on animal tuberculosis a number of laboratories responded to the questionnaire and indicated that they normally use between four and five slopes or vials of liquid medium to obtain 100mg of wet weight of cells for DNA extraction (Cousins *et al.*, 1996 - unpublished document). This problem was addressed in the course of this study, where different volumes of the modified Middlebrook 7H9 liquid medium were used to grow cells. As Figure 7.5 and Table 7.2 indicate, if the target is to obtain 100mg of cells then a 20ml volume was cost-effective compared to larger volumes, as there was a yield of 6.4mg/ml which is equivalent to 132.8mg per 20ml. Although larger volumes gave more weight per entire flask i.e. 265 and 470mg from 50 and 100ml respectively, the production of this amount is not cost-effective as weights per ml were lower than that produced by lower volumes (i.e. 7.1 and 6.4mg/ml from 10 and 20ml vs. 5.3 and 4.7 mg/ml in 50 and 100ml volumes). The difference observed in this experiment could be the outcome of the difference in ability to aerate different volumes of medium (Wayne 1976). In studies on growth of *M. tuberculosis* in liquid media, Wayne (1976) noticed that with continuous agitation the doubling time of *M. tuberculosis* was between 16 and 18 hours, while in unstirred medium there was a tendency towards an increased doubling time of nearly 33 hours. In the current study, flasks containing the cultures were shaken only once per week, for two main reasons; i) to aerate cells deposited on the bottom of the flask, and ii) to permit visual determination of growth. With this exercise, contents within the smaller flasks were well stirred and received better aeration than cells in the larger flasks.

A number of articles have indicated the use of Middlebrook 7H9 liquid medium for the isolation of *M. bovis* as well as *M. tuberculosis* (Cheng *et al.*, 1994; Skuce *et al.*, 1994/1996; Perumaallla *et al.*, 1996). None of these publications has indicated the

minimum time for growth but, the experience in our laboratory and an Australian laboratory (Dr. D. Cousins - personal communication 1996), has found that the minimum duration of incubation for *M. tuberculosis* was at least 4 weeks, while strains of *M. bovis* needed a much longer duration.

As far as the use of this medium in isolation of *M. tuberculosis* is concerned, Abe *et al.* (1992) and Cheng *et al.* (1994) have shown that Middlebrook 7H9 liquid medium was superior to L-J pyruvate and Middlebrook 7H11 media in recovery of mycobacteria from clinical specimens. The use of the modified Middlebrook 7H9 liquid medium described in the present work, for primary isolation of *M. bovis* from clinical material as well as comparison with other media was not addressed in the course of this study. It therefore should be a focus of future work on this medium.

The disadvantages of using liquid media for primary isolation is the inability to single out mycobacteria in a contaminated culture (Cheng *et al.*, 1994). In the present study two vials inoculated with ET3 and EB4 were found to be contaminated, representing a 10% contamination rate. This contamination might have been a carry over from the original culture, as the 2 tubes containing uninoculated modified media, remained free of any micro-organisms throughout the study period.

The chief advantages of using liquid media over solid media in molecular biology techniques are; i) the relative ease of harvesting the cells as compared to solid media, which need careful scraping of the growth from the surface of the media. This procedure does not guarantee freedom from particles of media, which results in false wet weights and in turn low yields of DNA upon extraction, ii) in the course of scraping the slope, the to and fro movements of the loop on the surface of the medium is likely to generate aerosols which are hazardous, and iii) it is easy to monitor contamination at an early stage of culture when liquid media is used, as the medium will be cloudy within a few days.

The results presented in this chapter have further confirmed the importance of sodium pyruvate for the growth of *M. bovis* and that the use of the modified Middlebrook 7H9 broth would offer an easy, safe and cost-effective approach in molecular studies requiring large amounts of DNA from *M. bovis*.

CHAPTER 8

GENERAL DISCUSSION

CHAPTER 8 GENERAL DISCUSSION

8.1. INTRODUCTION

It is over a hundred years since Dr. Robert Koch isolated and characterised the tubercle bacilli affecting cattle and man, but knowledge of the infectiousness of the disease between the two species preceded this by almost 40 years (Grange 1982). Although Klenkle (1846) described a clear association between the consumption of milk from infected cows and subsequent development of scrofula (tuberculous cervical lymphadenitis) (Collins and Grange 1983), it was not until the turn of this century that serious considerations were made regarding the pathogenicity of bovine tubercle bacillus for man (Francis 1959). The early evidence of this disease affecting man is shown in the tomb portraits of dynastic Egypt which showed people to be affected by Potts' disease, as early as 3000 BC (Cave 1939). Furthermore, it appears that present day *M. bovis* existed earlier than *M. tuberculosis* (Steele and Ranney 1958). In a review by these authors, it is stipulated that tuberculosis is believed to have affected animals that inhabited earth before the advent of man. Now it is known that bovine tuberculosis can be transmitted to man both by ingestion of milk from tuberculous cows, inhalation of bacilli in droplets expelled by animals' coughing, and to a lesser extent, through the consumption of meat from tuberculous animals (Sigurdsson 1945; Francis 1973; Grange and Yates 1994).

Following the recognition of the potential of *M. bovis* to cause disease in man and cattle, various countries instituted control measures to protect the human population. Measures undertaken included testing and slaughter of infected animals, pasteurization of milk and rigorous meat inspection at abattoirs (Collins and Grange 1983). These measures have reduced the incidence of the disease in many of the developed countries to below 0.1% (Steele 1995). However, the presence of the disease in a number of wildlife species (O'Reilly and Daborn 1995) compound the problem of achieving disease free status in some of the developed countries. In developing countries, the situation has not changed significantly. The majority of

these countries have no resources to implement test and slaughter control strategies (Alhaji 1977; Kleeberg 1984; Pritchard 1988), pasteurisation of milk is not compulsory (Kleeberg 1984) and the quality of meat inspection is poor due to the lack of properly trained meat inspectors (Alhaji 1977; Kazwala *et al.*, 1993).

Since the start of the HIV pandemic in the early 1980's, the numbers of tuberculosis cases encountered in various countries of the world has increased dramatically (Styblo 1989). This increase is not only confined to tuberculosis due to human tubercle bacillus but also with *M. bovis* (Bouvet *et al.*, 1993; Daborn and Grange 1993; Dankner *et al.*, 1993). An increased incidence of tuberculosis due to *M. bovis* has serious consequences not only to veterinarians but also to the medical profession. This is due to the following: first, a different approach is required to control infection in the general population; second, the epidemiological trace back of sources of infection to man; third, and most important, is that the management of *M. bovis* infection in man which is not identical to that of *M. tuberculosis*. *M. bovis* is naturally resistant to pyrazinamide (Konno *et al.*, 1967), hence, human infection by this organism cannot be treated effectively with the drugs currently prescribed in short course chemotherapy (SCC). In the SCC regime, pyrazinamide is used in combination with isoniazid hydrazide (INH) and rifampicin, and is regarded to be important in the treatment of tuberculosis as it kills intracellular susceptible tubercle bacilli as well as the semi-dormant tubercle bacilli that are not affected by other anti-tuberculosis drugs (Heifets and Lindholm-Levy 1992).

Apart from the zoonotic implication of the disease to human health, bovine tuberculosis is responsible for economic losses to farmers and businessmen. The cost of depopulation of herds in countries with eradication scheme is enormous not only to farmers but also the governments. Direct loss due to condemnation of organs, tissues and carcasses at post-mortem has been recorded to inflict up to 30% loss of returns from sales meat and its by-product in countries with high incidence of bovine tuberculosis (Francis 1972).

8.2. GENERAL EPIDEMIOLOGY OF BOVINE TUBERCULOSIS IN TANZANIA

Studies to determine the prevalence of the disease in cattle as well as man in Tanzania (Tanganyika) date back to the early 1920's, when a batch of dairy cattle imported from South Africa were found to have tuberculous lesions at post-mortem (Cornell 1934). It is also recorded that, in 1928, Medical Officers were reporting an increasing incidence of tuberculosis amongst people living in higher wetter areas, e.g. the Wachagga tribe residing on the slopes of Mount Kilimanjaro. This observation triggered a study to determine the presence of disease in their cattle, as the Wachagga people had a habit of sharing the same environment with their cattle in poorly ventilated houses. In that study, eight of 441 cattle tested positive, although none of four positive cattle that were necropsied had tuberculous lesions and it was concluded that the disease in cattle was of little consequence to human health (Cornell 1934). Several *ad-hoc* studies were performed between then and the late 1940's which showed the presence of disease in several foci within the country, but the worst affected area was the Southern Highlands (Macaulay 1957). Between 1949 and 1951 a thorough study was conducted by Markham, which found between 7 and 26% (average 11.3%) of indigenous zebu cattle grazing in the Southern Highlands, particularly the Usangu Plains, to be affected by bovine tuberculosis as determined by the single comparative intradermal test (Markham 1952). Markham could not attribute this high level of infection to any specific source. However, he was able to confirm the cause of infection to be due bovine tubercle bacillus by animal inoculations. Since this study, several other small studies have been carried out, particularly in grade or exotic cattle belonging to government farms (Table 1.12). There have not been any thorough attempts to determine the role of bovine tubercle bacillus as a cause of infection in human tuberculosis in Tanzania. Studies carried out by EA/BMRC (1975), T/BMRC (1985) and Ritcher *et al.* (1991) have indicated the isolation of *Mycobacterium* spp. from both pulmonary and extra-pulmonary forms of tuberculosis in man, but none of them reported isolation of *M. bovis*. Furthermore, the NTLP manual (1987) regarded *M. bovis* infection in man in Tanzania to be rare. However, since 1983, the total number of new cases of all forms

of tuberculosis started to rise and in a period between 1984 to 1995 the number rose from 12,092 to 39,847 per year, equivalent to an increase of 230%. During the same period, the proportion of cases of extra-pulmonary to total tuberculosis cases rose from 7.9% to 17% (NTLP report 1996). According to Kleeberg (1984), in situations where no efforts are made specifically to identify infections caused by *M. bovis* in man, a measure of this infection in a population could be suggested by the number of cases of extra-pulmonary tuberculosis, particularly cervical adenitis. This suggestion was taken into consideration when a nation wide survey was carried out in Tanzania (Catley 1992; Kazwala *et al.*, 1993). In that survey, a positive correlation ($r=0.67$) was found between keeping cattle and the proportion of cases of extra-pulmonary tuberculosis to total tuberculosis in man. This finding prompted the current study.

The current study, the main objective of which was to demonstrate the zoonotic importance of *M. bovis*, was designed to sample both cattle and man in two geographic zones; the Southern Highlands and the Arusha region in the north of Tanzania.

In the present study, three methods were used to determine infected animals; the single intradermal comparative tuberculin test (Lesslie and Herbert 1975), post-mortem diagnosis (Corner *et al.*, 1990) and culture of mycobacteria (Marks 1976; Watt *et al.*, 1993). Using these techniques, *M. bovis* infection in cattle in Tanzania was confirmed.

It was possible to detect a total of 911 (14.3%) of the 6383 cattle tuberculin tested in the Usangu Plains, while a total of 841 cattle were examined at post-mortem in several stations along the Usangu Plains and 225 (26.8%) had tuberculous lesions in their tissues. Pools of lymph node from the 841 animals slaughtered at the Usangu Plains and 1068 samples from another study in Arusha were submitted to SUA *Mycobacterium* laboratory for culture, although some ($n=190$) had to be discarded after being found to be putrefied before being processed. Therefore, a total of 1719 were cultured and 73 (4.2%) yielded *Mycobacterium* spp. A total of 805 milk samples also were processed and 36 (4.4%) yielded *Mycobacterium* spp. The overall proportion of *M. bovis* among the isolates which survived after primary isolation, was

38.9%. One strain was confirmed to be *M. tuberculosis* and the rest (60.2%) were atypical mycobacteria.

From an epidemiological point of view, the assessment of disease on a herd basis provides a better measure of the extent of the disease in a population rather than figures provided by the aggregate of animals in a large population (Thrusfield 1986). Using this criterion it was found that tuberculosis is wide spread in the plains, as 51% of the herds were found to be positive and there was a positive correlation ($r=0.85$) between herd level of infection and the size of the herd. Another important finding of the present study was the demonstration that zebu cattle were equally or more affected by the disease than exotic cattle, a finding which was in contrast with earlier reports (Carmichael 1938; Hornby 1949; Macaulay 1957; Waddington 1965) and in recent literature (Radostits 1994). The likely explanation for this difference is the different sample size examined. Those reports examined fewer cattle than that in the present study and a study by Markham (1952).

The present study also has shown the importance of atypical mycobacteria, which might greatly affect the performance of the tuberculin test (Karlson 1962). It should be noted that the majority (61/62) of these strains of atypical mycobacteria were from specimens collected from the Usangu Plains, where the number of cattle found to have an inconclusive reaction was almost double (28.5%) those classified as reactors. Furthermore, it raises the possibility that some of these atypical mycobacteria could be responsible for a proportion of the tuberculous lesions encountered at slaughter in this part of Tanzania. The presence of atypical mycobacteria in cattle could be linked with the ecology of the Usangu Plains. The presence of the Ruaha river in the middle of the plain would provide a suitable reservoir of saprophytic mycobacteria and the flooding of the plains during the rainy season would spread these mycobacteria throughout the plains. Alternatively, as has been speculated in other countries (Waddington 1965), birds feeding on aquatic animals from the river may be ingesting mycobacteria and spreading them on the grazing land via excreta.

The sensitivity (75%) and specificity (89%) of the tuberculin test, as applied in the Usangu Plains, was comparable to that reported by other studies (Francis *et al.*, 1978; Monaghan *et al.*, 1994). When the same test also was applied to a herd of 150 cattle

with no history of tuberculosis, belonging to the Sokoine University of Agriculture, none of these cattle reacted to the tuberculin test (data not shown) hence further confirming the specificity of the test as used in Tanzania.

One well-established route of transmission of *M. bovis* from cattle to man is through consumption of unpasteurised milk from infected cows (Sigurdsson 1945, Grange and Yates 1994; Chalmers *et al.*, 1996). The presence of only two (0.25%) *M. bovis* strains amongst 805 samples would seem negligible, but if milk from cows excreting *M. bovis* is mixed with that from non-infected cattle it will contaminate the pooled milk and pose a health risk to consumers in large numbers. A recent report of an outbreak of bovine tuberculosis in two herds of cattle in South West of Scotland, has revealed the public health consequence of consuming milk from an infected cattle (Chalmers *et al.*, 1996). In this outbreak, consumption of milk from a single cow was able to convert 2 of 5 people to Mantoux test positive. These people had no history of BCG vaccination (Chalmers *et al.*, 1996).

Regarding the respiratory route of infection (i.e. via aerosol droplets and droplet nuclei), it was not possible to take either nasal swabs or nasal discharges aseptically, this was due to the lack of established cattle restraint facilities. In the field, cattle were caste on the ground after a chase, this activity generated a lot of dust and/or mud in the kraals, which subsequently contaminated the nostrils and nasal passages of cattle. In view of these circumstances, the exercise was abandoned after a few attempts.

Regarding cultivation of mycobacteria from cattle specimens, this activity was greatly affected by the presence of contaminating bacteria, which necessitated using a longer decontamination time which in turn not only killed the contaminants but also severely affected the viability of mycobacteria present in the specimens. Extreme care was taken to minimise the cross contamination between carcasses; for each animal cleaned and disinfected knives were used and sterile scapel blades were used to slice pools of lymph nodes. However, local working conditions were such that contaminating bacteria might have arisen from slaughter and milking procedures. For example, slaughter was carried out on the floor of the slabs and the use of axes and

machetes in opening the carcasses could easily have led to mechanical contamination as well as generating aerosols containing bacteria from the environment.

In general, several tests used in the current study were able to demonstrate the presence of the disease in cattle. There was a strong agreement between the tuberculin test and post-mortem diagnosis. 70% of the reactor cattle were found to have visible lesions at enhanced meat inspection, an agreement which is important in gaining farmers confidence if a voluntary test and slaughter policy is to be performed. Simultaneous with the study in establishing the prevalence of tuberculosis in cattle, samples were collected from patients suffering from tuberculosis and residing in the same area. *M. bovis* was demonstrated in 7 (15.9%) of the 44 mycobacteria recovered from 149 cases studied. Six of the *M. bovis* isolates were recovered from patients with extra-pulmonary tuberculosis, while the remaining isolate was from a patient suffering from pulmonary tuberculosis. This finding concurred with suggestions made by Kleeberg (1984) that the main manifestation of *M. bovis* infection in man is the involvement of the extra-pulmonary organs. This proportion (15.9%) of *M. bovis* to total mycobacteria isolates is comparable to that reported elsewhere (Table 1.10). The isolation of several species of atypical mycobacteria has to be taken into consideration. Although some species are known to be of little consequence to human health (*M. terrae*, *M. flavescens* and *M. smegmatis*) others such as *M. avium*, *M. kansasii* and *M. fortuitum* are potentially pathogenic to man especially in immunocompromised individuals (Grange 1988). In the present study, data pertaining to HIV status was not provided, hence it was not possible to link these isolations to the immune status of the patient.

Transmission of *M. bovis* infection from cattle to in-contact people has been demonstrated not only in the past (Sigurdsson 1945) but also in the recent years. Chalmers *et al.* (1996) reported an outbreak of bovine tuberculosis in two herds of cattle in Dumfriesshire, Scotland, where six of the twelve people who had contact with infected cattle became Mantoux test positive and chemoprophylaxis was instituted upon them.

A study carried out in the village of Matebete failed to isolate any *M. bovis* from the pastoralists or milk samples collected from their cattle. However, *M. tuberculosis*

was isolated from two human cases, while *M. terrae* was isolated from both man and cattle on the same holding. The latter finding, apart from raising concerns of the aseptic condition of milk, also indicates a possible infection from a common environmental source or transmission between man and cattle. From the same geographic area, a pool of lymph nodes from one animal yielded *M. tuberculosis*. This observation raises the concern of public and animal health on the grounds that man being might have infected cattle, the so called “reverse zoonosis”, and also cattle reacting to bovine PPD might be due to sensitisation by both bovine and human tubercle bacilli. The current test is not specific enough to discriminate the two species. It has been reported in the past that *M. tuberculosis*, believed to be from cattle keepers/owners, caused sensitisation of cattle to the tuberculin test in attested herds (Lesslie 1960). However, it raises also concern about the possibility of mistaken identity in laboratory of the original strain. This speculation cannot be ruled out in any mycobacterium laboratory as has been reported elsewhere (Small *et al.*, 1993; Dunlap *et al.*, 1995).

The analysis of the epidemiological data accompanying cases from which *M. bovis* was isolated revealed several important observations; i) *M. bovis* infections were confined to people with cattle contact, which implies that these people might have acquired the infection from animal sources. Pastoralists, particularly the Maasai, have the custom of keeping younger stock indoors as well as drinking untreated raw milk and blood. These two activities provide a perfect scenario for transmission of infections from cattle to man. Similar observations had been made in Europe before the bovine tuberculosis eradication campaigns were instituted (Sigurdsson 1945), ii) *M. bovis* was more common in younger people. This finding is in agreement with what happened in the developed countries in the 1930’s to 1940’s where the disease affected children more than other age groups (Griffith and Munro 1935). The situation in the developed countries has changed due to the control measures instituted by these countries and now the disease is mainly confined to old people, and iii) an important observation made by this study was that patients, from whom *M. bovis* was isolated, had been vaccinated with *M. bovis* BCG. This finding indicates a lack of protection of the vaccine for people in this part of the world. A similar finding

was reported recently in Malawi, where a single BCG vaccine was not protective against tuberculosis (Karonga Prevention Trial 1996). As to why this vaccine does not work in Africa (see above) and in India (Anon 1980) could be linked with the following. First, other mycobacteria such as *M. avium* and *M. fortuitum* (Day 1996) may produce antigens that block the host immune system recognition sites for antigens from BCG vaccine, thus rendering individuals susceptible to subsequent *M. bovis* or *M. tuberculosis* challenge (Day 1996). The isolation of atypical mycobacterium from human cases might confirm this suspicion in Tanzania, but atypical mycobacteria are also found in countries which BCG vaccine is efficient (Murray 1990), therefore negates this supposition. Second, it is known that certain strains of *M. bovis* BCG (Glaxo, Tirc, Copenhagen and Pasteur) do not secrete MPB70 antigen (Radford *et al.* 1988). This protein, initially isolated from *M. bovis* BCG (Tokyo) (Fifis *et al.*, 1991), is the major immunoreactive antigen produced by all *M. bovis* strains (Radford *et al.*, 1988).

The isolation of *M. bovis* from both man and cattle is not adequate evidence that the infection is transmitted from one population to another. The biochemical tests used to identify this species (Watt *et al.*, 1993) are not equipped adequately to sub-type the strains of *M. bovis* and other members of the tuberculosis complex. No attempts were made to utilise conventional typing techniques, such as determination of antibiograms, phagetyping or serotyping (Grange 1988), as these techniques have been found to be ineffective and cumbersome to perform (Saunders 1995). Therefore, in this study, the recently developed DNA typing techniques were utilised to type the strains.

8.3. MOLECULAR EPIDEMIOLOGY

Molecular epidemiology is the integration of the molecular biology techniques in epidemiological studies intended to study the dynamics of infectious disease agents in populations. These techniques, which include restriction fragment length polymorphism (RFLP), have been used extensively in the typing of various micro-organisms including *M. tuberculosis* and *M. bovis* (Crawford 1993; Drobniewski *et al.*, 1994; Stanley and Saunders 1996). Prior to the application of these techniques to

field strains, it was important to evaluate their performance on a range of laboratory strains from various laboratories (Appendix IV). Hand-in-hand with the evaluation of RFLP, PCR protocols specific for the *M. tuberculosis* complex {i.e. IS986 PCR (Hermans *et al.*, 1990) and IS1081 PCR (Collins and Stephens 1991)}, *M. tuberculosis* (*mtp40* PCR - del Portillo *et al.*, 1991) and *M. bovis* (MPB70 PCR- Cousins and Wilton 1991; *M. bovis* specific PCR - Rodriguez *et al.*, 1995) also were evaluated in the current study. The results of PCR for IS986, IS1081 and *mtp40* were in agreement with the published accounts. However, the MPB70 PCR proved to be specific for the *M. tuberculosis* complex rather than *M. bovis* alone, hence it was not possible to single out *M. bovis* on the basis of any of the PCR protocols. The ability of IS986 RFLP to type strains of *M. tuberculosis* was confirmed as well as its inability to type strains of *M. bovis*. All the strains of *M. bovis* tested had a single copy of IS986 at the same chromosomal locus except one strain in which hybridisation occurring at 2.2Kb as opposed to 1.9Kb produced by the rest.

It was decided, therefore, to attempt and develop an alternative approach to typing and identification of *M. bovis* by molecular means. A PCR based technique termed intersegment PCR was devised. The decision to develop a PCR based technique emanated from the drawbacks of the commonly used DNA typing techniques that require culture of bacteria to yield at least 1-2µg of DNA and the technical sophistication of these techniques. The intersegment PCR was developed to exploit the polymorphism generated by the different positioning and numbers of the insertion elements IS986 and IS1081 in relation to each other, as well as to the single copy genes encoding *mtp40* and MPB70 antigens. This technique was able to produce polymorphic bands in unrelated strains by all seven permutations tested in this experiment. These results were by no means the result of the primers annealing specifically to sequences within the target DNAs. It was found that strains with a known single copy of IS986 produced more than a single band, strains of atypical mycobacteria, which were known not to contain any of the target DNA, also produced multiple bands with this technique. The technique could not be perfected further due to limitations of time. However, similar work was published when this work was at its early stages (Ross and Dwyer 1993) and another more recently

(Neimark *et al.*, 1996). These workers experienced similar non-specific amplification and upon sequencing of the PCR products they found that some of the products were due to primers annealing at homologous secondary structures within the genome. Both investigators (Ross and Dwyer 1993; Neimark *et al.*, 1996) advocated the use of this technique as an alternative for typing strains of *M. bovis* as well as low IS986 copy number *M. tuberculosis*. It is the opinion of the current study that applying this technique to field strains should be preceded by amplification of DNA elements specific for either the taxon or individual species as these primers also amplified DNA from atypical mycobacteria.

In a quest to obtain a *M. bovis* specific probe, the current study attempted to find a specific DNA sequence by cloning RAPD products which only were found in *M. bovis* and not in *M. tuberculosis* and other mycobacteria. Two fragments, termed *f2b* (accession number Z82981) and *f3b* (accession number Z82980) were successfully cloned into a pGEMT[®] vector. Their DNA sequence, and translated amino acid sequences matched one of the *M. tuberculosis* cosmids (accession number Z80343). This cosmid was found to have homology with the *rfbE* gene of *Yersinia enterocolitica* (accession number Z18920). These unique DNA elements were tested for their specificity for genomic DNAs from *M. bovis*, *M. tuberculosis* and atypical mycobacteria and found to be present in the *M. tuberculosis* complex and not in any of the atypical mycobacteria tested. The initial RAPD PCR favoured amplification of this product in *M. bovis*, but this event did not appear to occur by chance. In database searches, there were no matches of DNA sequences similar to that of the RAPD primer in region flanking the homologous sequence in the *M. tuberculosis* cosmid.

In view of these findings, it was not possible to apply any of the developed techniques on the field strains of *M. bovis* from Tanzania.

While the above experiments were in progress, a novel PCR based typing technique, termed spoligotyping (spacer oligonucleotide typing) was developed by Dutch scientists. This technique is capable of simultaneously detecting and typing not only strains of *M. bovis* but also other members of the *M. tuberculosis* complex (van Embden *et al.*, 1995). Therefore, this technique provided an alternative approach to type strains of *M. bovis*. Since this technique has not been used widely, it was

decided to utilise it in parallel with the internationally agreed standard RFLP (van Embden *et al.*, 1993). For the latter technique, two probes were used, IS986 for strains of *M. tuberculosis* and pTBN12 (Ross *et al.*, 1992) for *M. bovis*. pTBN12 is a plasmid containing a polymorphic GC rich sequence (PGRS) which has been found in multiple copies within the genome of strains of the *M. tuberculosis* complex (Table 1.5a). PGRS has been utilised to type single IS986 copy *M. bovis* BCG and *M. bovis*, as well as strains of *M. tuberculosis* with low IS986 copy number and was able to reveal polymorphisms among those strains (van Soolingen *et al.*, 1993). Regarding PCR to identify the *M. tuberculosis* complex, a multiplex PCR was able to identify correctly all 71 strains belonging to the *M. tuberculosis* complex. All atypical mycobacteria were negative. The *mtp40* PCR was in agreement with the biochemical tests in identifying *M. tuberculosis* in 27 of 36 isolates. Nine strains of *M. tuberculosis*, which were negative for *mtp40*, all came from the Usangu Plains, including a single strain from cattle. This finding concurs with recent observations made by Liebarna *et al.* (1996) and Weil *et al.* (1996). The specificity of *mtp40* for *M. tuberculosis* has been questionable not only because some strains *M. tuberculosis* do not contain this element, but some strains of *M. bovis* have been found to contain it (Romano *et al.*, 1995; van Embden 1995). The amplification of *mtp40* from strains of *M. africanum* (Vicent 1993; Liebarna *et al.*, 1996) casts further doubts on the specificity of this element for *M. tuberculosis*. These reports of lack of specificity of *mtp40* PCR are in contrast to Sinclair *et al.* (1995). The difference in these result might be attributed to differences in the primer sequences used by different groups rather than the absence of the gene. Second, the difficulty of differentiating these strains by biochemical tests could allot strains to different species, which would give false positive results. The *M. bovis* specific PCR (Rodriguez *et al.*, 1995) also was found not to be specific, as 35 of 37 strains of *M. tuberculosis* were positive. These results have highlighted further a need for evaluation of PCR on a wide range of strains of the *M. tuberculosis* complex before their specificity is declared in order to avoid misleading claims of specificity, such as occurred with *mtp40* and MPB70 PCRs. Strains of the *M. tuberculosis* complex are 98 to 100% homologous, and more so between *M. tuberculosis* and *M. bovis* (Imaeda 1985). This level of homology

makes it difficult to discriminate the two species. Some recent investigations on *pncA*, a gene encoding pyrazinamidase (Scorpio and Zhang 1996) and on the identification of polymorphic nucleotides in the *oxyR* gene in the *M. tuberculosis* complex (Sreevatsan *et al.*, 1996) have provided new knowledge on DNA elements which could be used to differentiate *M. bovis* from other strains of the *M. tuberculosis* complex. The work on the *pncA* gene has shown the presence of cytosine and guanine at nucleotide position 169 of *M. bovis* and *M. tuberculosis* respectively. A point mutation has been found in the *oxyR* gene of *M. tuberculosis* with guanine at nucleotide position 285 changed to adenine in *M. bovis* (Sreevatsan *et al.*, 1996). The demonstration of pyrazinamidase activity has been a key biochemical test in differentiating *M. bovis* and *M. bovis* BCG from the rest of the species of the *M. tuberculosis* complex (Grange 1990), while no specific role for the *oxyR* gene has been established in the *M. tuberculosis* complex (Sreevatsan *et al.*, 1996).

As regards identification of strains of *M. bovis* and *M. tuberculosis* by spoligotyping, in a majority of the strains the traditional bacteriologic identification was confirmed, but some *M. bovis* from man produced a unique RFLP and spoligotype which were so different to those found in cattle species that it could be speculated that there are strains of *M. bovis* which are circulating exclusively within the human population. As discussed above this observation concurs with the studies of van Embden *et al.* (1995), which found some unique strains of *M. bovis* from man that differed molecularly from those from cattle. But the observation made by van Embden *et al.* (1995) that some strains of *M. bovis* from Dutch patients which were *mtp40* positive and lacked the spacer sequence reminiscent of *M. bovis* would raise concern on their biochemical identification protocol. None of the *M. bovis* examined in the current study produced positive amplification of *mtp40*. Other workers also have encountered strains of *M. bovis* exhibiting properties similar to *M. tuberculosis* at the molecular level (Romano *et al.*, 1995) and suggested that these strains may represent a subtype which is only found in man with no connection with an animal reservoir.

Despite the above-mentioned shortcomings, spoligotyping has the advantages of speed, simplicity, versatility and is economical as it requires very little DNA to be performed. The blots (as provided by the inventor) can accommodate up to 45 strains

per single analysis and no sophisticated computer software is required for analysis of the spoligotypes.

Regarding molecular typing, this study has shown that *M. bovis* from cattle has infected man or vice versa because both the pTBN12 RFLP, IS986 RFLP and spoligotype patterns of some of *M. bovis* from man were similar to those produced by *M. bovis* from cattle when these typing probes/techniques were assessed individually (Figures 6.2, 6.5 and 6.9).

The genetic relatedness between these strains and those found in cattle ranged from as low as 43% to as high as 86% by pTBN12 RFLP typing, while with spoligotyping, these strains were genetically related to cattle strains by between 53 to 98%. These figures indicate a clonal relationship might have existed in the past. In a mass DNA typing of strains from various parts of the world it was found that strains of *M. bovis* with higher copy number of IS986 were those from wild animals, zoo animals or humans without contact with cattle (van Soolingen *et al.*, 1993). In the current study, two strains *M. bovis* from patients residing in Usangu had 13 and 15 copies of IS986. There was no matching number amongst 18 cattle strains typed by this probe. It is possible that these strains came from people who might have acquired that infection from wildlife. It should be noted that the Usangu Plains also encompass the Ruaha National park. This latter source was not investigated during the current study, but would be a focus of attention in future investigations of bovine tuberculosis in Tanzania.

There was geographical cross-over of DNA fingerprints exhibited by some isolates of *M. bovis* encountered in different parts of the study area, although the majority of DNA fingerprints were specific to strains from a given area (Figures 6.3, 6.6 & 6.10). This limited cross-over was expected in view of the fact that cattle kept by pastoralists in Tanzania are constantly moving in search of good grazing areas. In the course of their migration, some of the animals are sold for slaughter or their products are sold to local communities, creating a likelihood of infected cattle spreading the disease from one corner of the country to the other. The other explanation of this DNA fingerprint cross-over is that the human population is also dynamic as exhibited by the recent rural-urban migration of people due to the changing socio-economic

balance amongst people in Tanzania. This migration has resulted in overcrowding in towns and cities which has exacerbated the spread of many infectious diseases, particularly tuberculosis. The other important factor, is unrestricted livestock keeping in towns and cities. This activity would easily spread diseases such as tuberculosis from infected animals to urban people at a faster rate than in the rural areas for the following reasons: i) due to shortage of land, animals are sheltered in close proximity to their owners' living environment, ii) animal products particularly milk, are in high demand and are sold and frequently consumed, without treatment, iii) free-roaming cattle contaminate the environment, iv) inadequate waste disposal results in pollution of the environment. These factors would contribute significantly to the spread of the diseases not only to man but also from man to animals.

In the course of carrying out some of the molecular techniques (viz. Southern blotting, dot blotting) good quantities of unsheared DNA were required. This necessitated growing and harvesting approximately 100mg of cells. This weight was only achieved if several (~ 5) slopes of the L-J pyruvate and/or IUT were used. The growth of *M. bovis* in this medium is very slow, and hampered the speed of work. Also the procedure required a large amount of materials for growing of cells as well as enough incubator space. These problems were solved by the development of a modified Middlebrook 7H9 liquid medium. In this medium 0.4% sodium pyruvate was added to enhance the growth of *M. bovis*. It was therefore possible to grow 100mg of cells within three weeks by the use of 20ml of this medium inoculated with up to 10^5 cfu/ml of log phase culture. This medium could be of benefit for the initial cultivation of *M. bovis* from clinical specimens.

8.4. CONCLUDING REMARKS

The current study has demonstrated for the first time the zoonotic importance of *M. bovis* infection in Tanzania. Despite the few strains recovered from man, similar genetic fingerprints were demonstrated among those strains recovered from man and cattle in the study areas, which indicated the spread of the disease in the country. The study also reaffirmed the importance of molecular techniques in epidemiological

studies of infectious diseases such as tuberculosis which can cross species barriers. The isolation of *M. bovis* from animal products such as milk and meat has once more alerted public health authorities to the need for enforcement of laws governing public safety. In Tanzania, meat inspection is conducted under meat hygiene ordinances, but its enforcement needs careful assessment in light of the finding of many granulomatous lesions caused by non-tuberculous micro-organisms. Pasteurisation or any form of milk treatment has never been compulsory in Tanzania, but would require careful consideration in the wake of the HIV pandemic where many people who are immuno-compromised, might succumb to not only pathogenic mycobacteria but also to atypical mycobacteria. Tuberculin testing would still be the test of choice for live cattle as it has been used successfully in eradication campaigns conducted in developed countries. However, due to a low specificity and sensitivity, a need to develop/utilise newer techniques for direct detection of *M. bovis* in live cattle would be ideal in situations where a voluntary test and slaughter policy is to be implemented. Control of bovine tuberculosis in both man and animals in Tanzania would be achieved if the following are considered: i) increased awareness of the population to the dangers posed by infected animals, this can be achieved by public health education, ii) an improvement of public health infrastructure and its management (e.g. slaughter slabs in rural areas and slaughter houses in towns and cities), iii) development of improved techniques for the direct detection of infection in animals at the farm level, iv) enforcement of laws governing public safety, i.e. proper meat inspection procedures, proper disposal of condemned materials and restrictions on livestock keeping in the towns and cities, v) introduction of a vaccination campaign to areas known to be foci of infection e.g. the Usangu Plains, and vi) introduce/improve a veterinary medical approach in controlling the disease. The present study has once more reiterated the pivotal role that can be played by molecular techniques in epidemiological studies of infections caused by the *M. tuberculosis* complex. Their application in Tanzania has been the beginning of a new era in research of tuberculosis both in man and animal. Prior to these studies, no efforts, were made to determine the sources of tuberculosis in man particularly the role played by the animal derived mycobacteria. While this work was in progress, the

author and other members of the BTB project were invited to present some of the preliminary results of this work to the health authorities of Tanzania. As a consequence of that, the NTLP is revising its manual to re-address the role of *M. bovis* in human tuberculosis in Tanzania. Furthermore, preliminary findings of the present study have been presented to various other scientific gatherings. Although it could be argued that the molecular techniques utilised/developed during the current study cannot be afforded by Tanzania, it should be noted their use in epidemiological studies will outweigh the costs currently incurred in the treatment of new cases of tuberculosis each year. In Tanzania, the NTLP's main line of control of tuberculosis is finding new cases and the institution of chemotherapy. This approach will take many years to control the disease in light of the current upsurge of cases of HIV in the human population. Hitherto a need to institute measures to control the sources of the infection will deliver a cost-effective control strategy. In order to achieve that, the tuberculosis control strategy in Tanzania will need to be directed towards case finding, establishment of the sources and control the infection at its origin (the traceback approach). As it has been shown by a mass of recent reports, tracebacks of mycobacteria infections are not easily achieved by the use of the conventional techniques (Crawford *et al.*, 1993; Drobniewsk *et al.*, 1994; Saunders 1995; Stanley and Saunders *et al.*, 1996). The only available approach is the use of molecular techniques such as those described in the present study.

The contribution of knowledge on the role of *M. bovis* from animals to man made by the current study, though, small but opens a new era in control of the disease in Tanzania.

It is worth drawing a quote from Torning (1965) to the attention of many people involved in the control of tuberculosis, particularly in developing countries. "If tuberculosis is eradicated from the mind of the doctors, if only for a short time, its eradication from the bodies of their patients will be deplorably postponed".

Therefore the proposed action plan to tackle bovine tuberculosis and especially zoonotic aspects, in Tanzania need to address the following;

i) The study of the zoonotic importance of *M. bovis* in man requires further investigation, therefore an extension of the study to the areas beyond those covered

by the present study is appealing. In the current study, samples of extra-pulmonary tuberculosis were the only ones collected from Arusha, therefore it would make a more complete study if an assessment is also made of pulmonary involvement of *M. bovis*. In this region there are people who live indoors with their cattle up to the present day. Similarly, more cases of extra-pulmonary tuberculosis need to be examined in the Usangu Plains to rule out this infection in this form of the disease.

ii) Any success of this work will require the whole hearted participation of the medical profession. Establishment of a veterinary/medical liaison group for the research on zoonotic diseases would be a first step to control of many diseases of such a nature.

iii) A need to assess the potential role played by other domestic animals and wild animals species as reservoirs of this infection.

iv) Development of a specialised/reference laboratory with molecular capability in detection and typing of *M. bovis* and other pathogenic mycobacteria.

v) Establish a surveillance system to monitor the spread of the disease from animals to man and vice versa. This would be achieved by creating a database of DNA profiles of strains of *M. bovis* circulating between the two populations.

vi) Conduct a pilot vaccination trial to assess the efficacy of the *M. bovis* BCG or *M. vaccae* vaccines in Tanzanian cattle for the purpose of designing a less expensive approach in controlling bovine tuberculosis in Tanzania.

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APPENDIX I - RECORDS OF CATTLE TUBERCULIN TESTED

Questionnaire Number _____
Ear Tag Number _____
Check Letter _____
Date _____
Slab _____
Origin _____
HerdID _____
Breed _____
Permanent Incisor Pairs _____
Sex _____
Calving Last _____
 Next _____
Lactating _____
Tuberculin _____
Initial Reading Avian ____ Bovine ____
Repeat Stimulation _____
24 hour Reading Avian ____ Bovine ____
48 hour Reading Avian ____ Bovine ____
72 hour Reading Avian ____ Bovine ____
96 hour Reading Avian ____ Bovine ____
120 hour Reading Avian ____ Bovine ____
Notes _____

APPENDIX II - QUESTIONNAIRE FOR HUMAN CASES OF TUBERCULOSIS

BOVINE TUBERCULOSIS IN THE TROPICS STUDIES ON ZOONOTIC IMPLICATION OF *M. BOVIS*

BACKGROUND INFORMATION

Mycobacterium bovis is a member of *Mycobacterium tuberculosis* complex organisms. The pathogen is mainly found in cattle where it is responsible for pulmonary tuberculosis. However, *M. bovis* may cause infection in man, resulting to both pulmonary and extra-pulmonary tuberculosis.

It is therefore the purpose of this study to investigate the mode of transmission to man.

IDENTIFYING INFORMATION

Questionnaire Number _____

Date of Interview _____

Region _____

District _____

Hospital _____

Clinic _____

Patient's last name _____ First name & initial _____

Patient's code number _____

Address _____ (ten cell leader)

Name of person supplying information (if not named above) _____

GENERAL INFORMATION

Confounding variables

- i) Age ### years or (if under 2 yrs) age in months ##
- ii) Sex ____ (M/F)
- iii) Tribe _____
- iv) Marital status _____
- v) Educational status _____
- vi) Family size ##
- vii) Number of children ## Boys ## Girls ##
- viii) Children - less than 5 years ##
- ix) Children - more than 5 years ##

EXPOSURE TO FACTORS WHICH MAY INFLUENCE HEALTH

a) State of health

- i) When did you first notice signs of illness _____
- ii) What were the initial signs _____
- iii) When did you first report to the medical officer _____
- iv) When did you receive first treatment _____
- v) Did you have related illness in the past _____
- vi) Was it treated and cured ____ (Y/N)
- vii) Did you have BCG ____ (Y/N)
- viii) In your family who had BCG, Adults ____ Children ____ (Y/N)

b) Quality of housing

- i) Describe the house you live in - walls _____
- ii) Do you use cow dung to plaster walls ____ (Y/N)
- iii) Type of roof _____ type of floor _____
- iv) How many rooms ##
- v) How many people per room ## or ##
- vi) How many windows per room ##
- vii) Does the house have the toilet - Indoors ____ Outdoors ____ (Y/N)
- viii) If outdoors how far from the house (meters) ##
- ix) Do you keep animals in your house at night, ____ (Y/N) specify _____

c) Occupation and contact with animals

- i) In your village what is the main occupation _____
- ii) Do you keep cattle ____ (Y/N) since when _____
- iii) How many cattle do you have - 1 - 5 ____ 6-20 ____ above 20 ____ (Y/N)
- iv) Where are cattle housed _____
- v) How far from living area _____
- vi) How is manure disposed of _____
- vii) How many cows in your herd are being milked _____
- viii) What is your role in keeping cattle _____
- ix) For how long have you been doing this _____
- x) What other animals do you own: Pigs ____ Goats ____ Sheep ____
- xi) Chickens ____ others specify _____ (Y/N)

d) Consumption of animal products

- i) Are you an habitual milk drinker ____ (Y/N)
- ii) What is the source of milk you drink _____
- iii) If milk is from your cattle, who does the milking _____
- iv) Which kind of container is used for milking _____
- v) How is the container cleaned _____
- vi) Does fresh milk undergo any sort of treatment ____ (Y/N) mention _____
- vii) Do you take sour milk ____ (Y/N) how is it prepared _____
- viii) Do you take any other dishes where milk is one of the component _____
- ix) What is the source of meat you consume _____
- x) Does the meat inspector examine slaughtered carcass before the meat is distributed to the public ____ (Y/N)
- xi) In which state do you prefer your
 - meat for consumption _____
 - offals „ „ _____
 - blood „ „ _____

e) Sharing of eating and drinking utensils in the family and others

- i) As a family do you have communal meals on trays ____ (Y/N)
- ii) Is water readily available for the domestic use ____ (Y/N)
- iii) Source of water/any treatment _____
- iv) How many containers are used to store drinking water _____
- v) Is the cup used for drinking water normally shared by the family ____ (Y/N)
- vii) How do you wash your hands before eating _____
- viii) Do you take local brew ____ (Y/N) share with others ____ (Y/N)

OTHER INFORMATION

Have any family members suffered from tuberculosis before ____ (Y/N)

CLINICAL EXAMINATION (by physician)

Suspected Pulmonary tuberculosis ____ Extra pulmon. tb ____
ENT exam (NOR/ABNOR)____ Head/Neck exam (NOR/ABNOR) ____ Specify

Diagnosed at hospital/clinic Pulmonary or Extra-pulmonary tuberculosis _____

LABORATORY DIAGNOSIS

Mycobacterium spp. isolated _____

APPENDIX III - MINI QUESTIONNAIRE FOR HUMAN CASES OF TUBERCULOSIS

Questionnaire Number _____
Date of Interview _____
Hospital/Clinic _____
Clinic _____
Patient name _____
Age _____
Sex ____ (M/F)
BCG scar ____ (Y/N)
Do you keep animals in your house at night _____
In your village what is the main occupation _____
Do you keep cattle ____ (Y/N)
How many cattle _____
Are you an habitual milk drinker ____ (Y/N)
What is the source of milk you drink _____
Do you take sour milk _____
Sharing of eating and drinking utensils in the family _____

APPENDIX IV - THE MYCOBACTERIA STRAINS DONATED BY LABORATORIES FROM VARIOUS COUNTRIES.

Strains of mycobacteria used in the evaluation of molecular identification and typing techniques (Chapter 4).

	Origin	Species	MRI No.		Origin	Species	MRI No.
1*	Australia	<i>M. bovis</i>	MB14	26	Holland	<i>M. tuberculosis</i>	MT19
2*	Australia	<i>M. bovis</i>	MB15	27	Holland	<i>M. tuberculosis</i>	MT20
3*	Australia	<i>M. bovis</i>	MB16	28	Holland	<i>M. tuberculosis</i>	MT21
4	Australia	<i>M. bovis</i>	MB17	29	Holland	<i>M. tuberculosis</i>	MT22
5*	Australia	<i>M. bovis</i>	MB18	30*	Holland	<i>M. tuberculosis</i>	MT23
6*	Australia	<i>M. bovis</i>	MB19	31	Scotland	<i>M. gordonae</i>	MOTT1
7	Australia	<i>M. bovis</i>	MB20	32	Scotland	<i>M. marinum</i>	MOTT2
8*	Australia	<i>M. bovis</i>	MB21	33	Scotland	<i>M. kansasii</i>	MOTT3
9*	Australia	<i>M. bovis</i>	MB24	34	Scotland	<i>M. malmoense</i>	MOTT4
10*	Australia	<i>M. bovis</i>	MB25	35	Scotland	<i>M. fortuitum</i>	MOTT5
11*	Australia	<i>M. bovis</i>	MB26	36	Scotland	<i>M. tuberculosis</i>	MT3
12*	Holland	<i>M. bovis</i>	MB9	37	Scotland	<i>M. tuberculosis</i>	MT5
13*	Holland	<i>M. bovis</i> BCG	MB12	38	Scotland	<i>M. tuberculosis</i>	MT7
14*	Holland	<i>M. bovis</i> BCG	MB13	39*	Tanzania	<i>M. tuberculosis</i>	MT1
15	Holland	<i>M. africanum</i>	MAFR1	40*	Tanzania	<i>M. tuberculosis</i>	MT25
16	Holland	<i>M. africanum</i>	MAFR2	41*	Tanzania	<i>M. tuberculosis</i>	MT26
17	Holland	<i>M. tuberculosis</i>	MT8	42*	Tanzania	<i>M. tuberculosis</i>	M6051
18	Holland	<i>M. tuberculosis</i>	MT9	43*	Tanzania	<i>M. tuberculosis</i>	MT28
19	Holland	<i>M. tuberculosis</i>	MT10	44*	Tanzania	<i>M. tuberculosis</i>	MT29
20	Holland	<i>M. tuberculosis</i>	MT11	45*	Tanzania	<i>M. tuberculosis</i>	MT30
21	Holland	<i>M. tuberculosis</i>	MT12	46*	Tanzania	<i>M. tuberculosis</i>	MT31
22	Holland	<i>M. tuberculosis</i>	MT13	47*	Tanzania	<i>M. tuberculosis</i>	MT32
23	Holland	<i>M. tuberculosis</i>	MT14	48*	Tanzania	<i>M. tuberculosis</i>	MT33
24	Holland	<i>M. tuberculosis</i>	MT15	49	Tanzania	<i>M. bovis</i>	MB27
25	Holland	<i>M. tuberculosis</i>	MT17				

* Strain involved in IS986 RFLP typing.

A multiplex polymerase chain reaction for distinguishing *Mycobacterium tuberculosis* from *Mycobacterium tuberculosis* complex

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A multiplex polymerase chain reaction has been developed which is able to distinguish *Mycobacterium tuberculosis* from other members of the *M. tuberculosis* complex. The assay is based on the simultaneous amplification of two different targets: a 396bp region from the mtp40 species-specific gene sequence of *M. tuberculosis* and a 245bp fragment from the *M. tuberculosis* complex insertion sequence IS986. Results have been obtained for 54 mycobacterial strains including five non-*M. tuberculosis* complex isolates. All 49 strains of the *M. tuberculosis* complex were positive for IS986 but only the 27 *M. tuberculosis* isolates were positive for both IS986 and mtp40.

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KEYWORDS: *M. tuberculosis* complex, polymerase chain reaction, diagnosis.

INTRODUCTION

Many aspects of tuberculosis in both animals and man have been investigated extensively, yet tuberculosis remains the single greatest cause of morbidity and mortality in man.^{1,2} *Mycobacterium tuberculosis* is the most frequent cause of human tuberculosis, but other members of the *M. tuberculosis* complex, particularly *M. bovis*, may be involved.³ In developing countries, where *M. bovis* infection of several animal species can be widespread, the distribution, epidemiology and zoonotic implications of this infection are unknown. It is important, therefore, to identify and type accurately all tubercle bacilli isolated from patients and animals as this has implications for control programmes as well as for patient therapy.

Members of the *M. tuberculosis* complex may be identified and characterized in a number of ways. Cultural and biochemical tests, though useful, require

pure cultures of the isolated mycobacterium and are low in sensitivity.^{4,5} Other more sophisticated procedures such as thin layer chromatography,⁶ high performance liquid chromatography,⁷ pyrolysis mass spectroscopy,⁸ nucleic acid hybridization and restriction fragment length polymorphism⁹ are slow, not readily available and/or do not adequately discriminate between members of the *M. tuberculosis* complex. On the other hand, the polymerase chain reaction (PCR) has the potential to detect a single mycobacterial cell in only a few days.¹⁰ Within the mycobacteriaceae, several target sequences have been identified allowing detection of all mycobacteria,¹¹ members of the *M. tuberculosis* complex,^{9,12} or only *M. tuberculosis*.¹³

This paper describes a multiplex PCR reaction combining amplification of the *M. tuberculosis* complex

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Table 1. Mycobacterial strains used in this study

	Number	Origin
<i>M. tuberculosis</i> complex		
<i>M. tuberculosis</i>	10	Tanzania ¹
	13	The Netherlands ²
	4	Scotland ³
<i>M. bovis</i>	2	The Netherlands ²
	1	Scotland ⁴
	4	Scotland ³
	11	Australia ⁵
<i>M. bovis</i> BCG	2	The Netherlands ⁶
<i>M. africanum</i>	1	The Netherlands ²
	1	The Netherlands ⁶
Other mycobacteria		
<i>M. kansasii</i>	1	Scotland ³
<i>M. malmoense</i>	1	Scotland ³
<i>M. goodii</i>	1	Scotland ³
<i>M. marinum</i>	1	Scotland ³
<i>M. smegmatis</i>	1	Scotland ⁴

1. Sokoine University of Agriculture, Tanzania.

2. van Soolingen *et al.*, 1991.

3. Scottish Mycobacteria Reference Laboratory, Edinburgh.

4. Moredun Research Institute, Edinburgh.

5. Australian Reference Laboratory for Bovine Tuberculosis, South Perth.

6. Royal Tropical Institute, Amsterdam.

specific IS986⁹ and the *M. tuberculosis* specific mtp40.¹³ The procedure detects all members of the *M. tuberculosis* complex and simultaneously identifies and discriminates *M. tuberculosis*, avoiding the need for further tests.

MATERIALS AND METHODS

Mycobacterial strains

Forty nine strains of the *M. tuberculosis* complex and one each of five other species were used (Table 1). Each strain was tested for the presence or absence of IS986 and mtp40 by separate PCRs. All mycobacterial strains were grown on both IUT and pyruvate media.

Growth from each organism was removed from the medium with a sterile toothpick and resuspended in 40 µl of sterile deionized water. The suspension was heated to 95°C for 15 min, vortexed briefly and centrifuged (13 500 *g* for 30 s) prior to using 2 µl in a 50 µl PCR reaction.

Oligonucleotide primers

Four pairs of primers were synthesized by Oswel DNA, Edinburgh. The sequences and primer locations

are listed in Table 2. Primers F289 and F290 amplify a region of IS986 internal to INS-1 and INS-2. F538 and F539 amplify a region of mtp40 internal to PT1 and PT2.

Polymerase chain reaction

Reactions were done in 50 µl volumes containing 50 mM KCl, 2.5 mM MgCl₂, 10 mM Tris-HCl pH 8.0, 0.01% gelatin, 100 µM each dNTP (Pharmacia), 0.5 U *Taq* polymerase (Boehringer Mannheim) and 0.1 µM of each primer.

In the multiplex PCR, 0.05 µM of INS-1 and INS-2 and 0.1 µM of PT1 and PT2 were used per 50 µl reaction.

RESULTS

Only the 49 strains of the *M. tuberculosis*-complex were positive for IS986, producing a 245 bp product when amplified with primers INS-1 and INS-2. *M. malmoense*, *M. kansasii*, *M. goodii*, *M. smegmatis* and *M. marinum* were negative. Of the 54 mycobacterial strains tested for *M. tuberculosis* mtp40 using the primer pair PT1 and PT2, only the 27 *M. tuberculosis* strains were positive.

The conditions for the multiplex PCR, combining the primer pairs for IS986 and mtp40, were optimized by varying the annealing temperature and the concentrations of MgCl₂, dNTPs and primers (data not shown). The optimized multiplex PCR was then used to examine a panel comprising four strains of *M. bovis* and four of *M. tuberculosis*. Each of the *M. bovis* strains yielded only a single 245bp product whereas each of the *M. tuberculosis* strains yielded a 245bp product and a larger product of 396bp (Fig. 1). The sizes of these products were accorded with those predicted for IS986 and mtp40, respectively, and their identity was confirmed by hybridization using labelled internal probes generated by PCR using primers F290/F289 for IS986 and F538/F539 for mtp40 (data not shown). The remaining 46 mycobacterial isolates were tested by the multiplex PCR (Table 3). All members of the *M. tuberculosis* complex were positive for IS986, but only the 23 *M. tuberculosis* strains were positive for mtp40. *M. kansasii*, *M. goodii*, *M. marinum*, *M. smegmatis* and *M. malmoense* were negative for both.

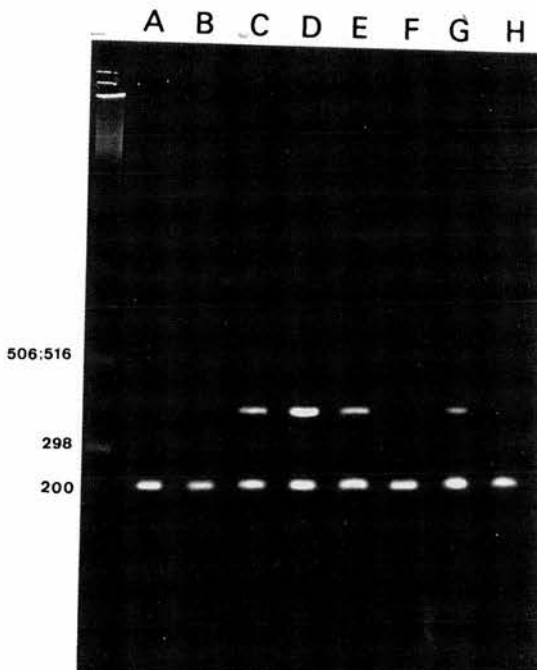
The reactions were subjected to 30 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 60 s in a Techne PHC-3 thermocycler. After amplification, 5 µl of loading buffer was added to the reactions and 20 µl loaded

Table 2. Oligonucleotide primers used in this study

Primer	Size (bp)	Target	Sequence (5'–3')	Location (bp)
INS-1	245	IS986	CGTGAGGGCATCGAGGTGGC	631–650 ¹
INS-2	245	IS986	GCGTAGGCGTCGGTGACAAA	856–875 ¹
F290	205	IS986	CAGATGCACCTGCGAACGGCT	651–671 ²
F289	205	IS986	GGCCACGTAGGCGAACCCCTGC	835–855 ²
PT1	396	mtp40	CAACGCGCCGTCGGTGG	9–25 ³
PT2	396	mtp40	CCCCCACGGCACCGC	385–401 ³
F538	341	mtp40	AACACCACGTTAGGGATG	31–48 ²
F539	341	mtp40	TCACGGTGCCCAACATCG	347–364 ²

1. McAdam *et al.*, 1990.

2. This study.

3. Del Portillo *et al.*, 1991.**Fig. 1.** Amplification of *Mycobacterium* species by multiplex PCR. Samples were amplified for 30 cycles using INS1 + INS2 and PT1 + PT2 primer pairs. (A) *M. bovis* 28050, (B) *M. bovis* 9640, (C) *M. tuberculosis* 22604, (D) *M. tuberculosis* 21928, (E) *M. tuberculosis* 20963, (F) *M. bovis* 25833, (G) *M. tuberculosis* 18186, (H) *M. bovis* 34360.

onto a 2% agarose gel. The products were visualized by ethidium bromide fluorescence.

DISCUSSION

The findings of this study complement the original observation that mtp40 is present only in *M. tuberculosis*^{13,16} and is absent in 14 other species of mycobacteria. This specificity of mtp40 makes it a

Table 3. Results of multiplex PCR on 46 mycobacterial strains

	No. of strains tested	IS986	mtp40
<i>M. tuberculosis</i> complex			
<i>M. tuberculosis</i>	23	+	+
<i>M. bovis</i>	14	+	–
<i>M. bovis</i> BCG	2	+	–
<i>M. africanum</i>	2	+	–
Other mycobacteria			
<i>M. kansasii</i>	1	–	–
<i>M. malmoense</i>	1	–	–
<i>M. goodii</i>	1	–	–
<i>M. marinum</i>	1	–	–
<i>M. smegmatis</i>	1	–	–

suitable target for use in the development of a diagnostic test for *M. tuberculosis*.

Various PCR-based protocols have been published for the identification of the *M. tuberculosis* complex based on the amplification of specific sequences.^{9,17} The advantages of these techniques are their speed and sensitivity but, in general, the sequences amplified do not discriminate within the *M. tuberculosis* complex. Plikaytis *et al.* (1991) developed a method for the differentiation of *M. tuberculosis* and *M. bovis* BCG based on the differing number of copies of IS6110 in each organism.¹⁸ *M. tuberculosis* contains 10–16 copies and *M. bovis* BCG 3–6 copies of IS6110 and this method relied on the copy number of IS6110 remaining constant. The discovery, however, of *M. tuberculosis* isolates with fewer than 10 insertional elements placed some doubt on the reliability of this test.¹⁴ The PCR described in this report amplifies a single copy sequence, mtp40, determined to be specific for *M. tuberculosis*, in addition to a *M. tuberculosis* complex sequence, IS986. This technique therefore will reliably identify *M. tuberculosis* and

discriminate it from other members of the *M. tuberculosis* complex. The inclusion of IS986 provides not only an internal control to confirm that amplification has occurred, but also, in the absence of an mtp40 signal, detects other members of the *M. tuberculosis* complex, namely, *M. bovis*, *M. microti* or *M. africanum*.

M. tuberculosis is the most frequent cause of human tuberculosis but other members of the *M. tuberculosis* complex, particularly *M. bovis* may be involved. Although the exact importance of *M. bovis* in human infections is still unclear, the proportion of human tuberculosis resulting from *M. bovis* infection has been reported as ranging from 2.7% in the south east of England¹⁹ to 3.8% in Buenos Aires City.²⁰ A recent world-wide survey of 17 laboratories (A. Fanning, personal communication) revealed that most laboratories employed a glycerol-containing medium for primary isolation. Since glycerol suppresses growth of *M. bovis* the use of such medium could underestimate the incidence of *M. bovis* infection.²¹

A definitive diagnosis is necessary also when considering chemotherapy. *M. bovis* is intrinsically resistant to the antimycobacterial pyrazinamide. Failure to discriminate the resistant *M. bovis* from the sensitive *M. tuberculosis* would influence the effectiveness of the treatment and could threaten patient response. The application of PCR directly to clinical samples²² or following extraction of mycobacteria²³ has been shown to be effective in the diagnosis of tuberculosis. The use of the multiplex PCR described here in the diagnosis of tuberculosis would provide a faster and more sensitive method of detection, in addition to discriminating between members of the *M. tuberculosis* complex. This is important when considering chemotherapy and, also, the results would provide a better understanding of and more reliable information on *M. tuberculosis* infection and *M. tuberculosis* complex infection.

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